CHARACTERIZATION OF THE CLINICAL, HISTOLOGICAL AND GENETIC PROFILE OF ARTICULAR DAMAGE IN HEREDITARY HEMOCHROMATOSIS

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Tese para obtenção do grau de Doutor em Medicina
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(Assinatura)

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Acronyms

3D three dimensional.

Acan aggrecan.

ACC articular calcified cartilage.

ADAMTS5 a disintegrin and metallopeptidase with thrombospondin motifs 5.

Adamts5 a disintegrin-like and metallopeptidase with thrombospondin type 1 motif, 5.

BMD bone mineral density.

Bmp6 bone morphogenetic protein 6.

C282Y cysteine-to-tyrosine substitution at amino acid 282.

cDNA complementary deoxyribonucleic acid.

CI Confidence Interval.

Col10a1 collagen, type X, alpha 1.

Col2a1 collagen, type II, alpha 1.

DALYs disability-adjusted life years.

DEXA dual-energy X-ray absorptiometry.

dGEMRIC delayed gadolinium-enhanced MRI of the cartilage.

ECM extracellular matrix.

EDTA ethylenedinitrilotetraacetic acid.

FPN ferroportin.

GAG glycosaminoglycan.

H63D histidine-to-aspartic acid substitution at position 63.

HA hyaluronic acid.

HAC hyaline articular cartilage.

HAMP hepcidin antimicrobial peptide.

HFE hemochromatosis.

Hfe hemochromatosis.

HH Hereditary Hemochromatosis.

HIC hepatic iron concentration.

HJV hemojuvelin.

HKA hip-knee-ankle.

HLA human leukocyte antigen.

IL1 interleukin 1.

Il1b interleukin 1 beta.

Il6 interleukin 6.

IQR interquartile range.

KBD Kashin-Beck disease.

KL Kellgren-Lawrence.

LBA load-bearing axis.

M-MLV Moloney Murine Leukemia Virus.

MHC major histocompatibility complex.

MMP13 matrix metallopeptidase 13.

Mmp13 matrix metallopeptidase 13.

MMP3 matrix metallopeptidase 3.

Mmp3 matrix metallopeptidase 3.

MRI magnetic resonance imaging.

mRNA messenger RNA.

NHANES National Health and Nutrition Examination Survey.

NSAID non-steroidal anti-inflammatory drugs.

NTBI non-transferrin bound iron.

OA osteoarthritis.

OARSI Osteoarthritis Research Society International.

OP osteoporosis.

PBS phosphate buffer saline.

RNA ribonucleic acid.

ROI region of interest.

ROS reactive oxygen species.

Rpl13a ribosomal protein L13A.

RQI RNA quality indicator.

RT reverse transcription.

RT-PCR real-time polymerase chain reaction.

Runx2 runt related transcription factor 2.

TBS Tris buffered saline.

TFR2 transferrin receptor 2.

Tfrc transferrin receptor.

TNF-α tumour necrosis factor α.

VCAM-1 vascular adhesion molecule 1.

WT wild-type.

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Summary

Osteoarthritis (OA) is the most common joint disease in humans. It affects the joint as a whole and is characterized by progressive articular cartilage destruction, abnormal subchondral bone remodelling, formation of osteophytes, ligament and periarticular muscle weakening and in some cases synovial inflammation, which ultimately lead to a painful and impaired joint. There are several known risk factors for the development of OA but the exact sequence of events that lead to the destruction of the articular cartilage is not yet fully understood.

Hereditary hemochromatosis (HH), a disease caused by mutations in the HFE gene, is characterised by systemic iron overload, toxic accumulation of iron in parenchymal cells of liver, heart, and endocrine glands. It is also associated with musculoskeletal complications, namely an increased prevalence of OA. The role of iron overload in the development of OA is still undefined.

To further understand the molecular mechanisms involved in the pathology of HH-related OA, we surgically induced OA in the knee of a murine model of hereditary hemochromatosis and studied the changes to cartilage and bone. Also, in order to understand how the different mutations in the HFE gene affect systemic iron overload and related musculoskeletal complications, we studied the prevalence of musculoskeletal complications in a cohort of patients with different HH genotypes.

Hfe-KO mice showed a systemic iron overload and an increased iron accumulation in the knee synovial membrane following surgery. The histological OA score was significantly higher in the Hfe-KO mice at 8 weeks after surgery. Micro-CT study of the proximal tibia revealed increased subchondral bone volume and increased trabecular thickness. Gene expression and immunohistochemical analysis showed a significant increase in the expression of matrix metallopeptidase 3 in the joints of Hfe-KO mice compared with control mice at 8 weeks after surgery.

Among our cohort of HH patients the majority were homozygous for the C282Y mutation. The serum ferritin concentration and serum transferrin saturation at diagnosis were significantly higher in C282Y homozygous patients compared with those who were compound heterozygous (C282Y/H63D). Also the overall prevalence of self-reported musculoskeletal complications was significantly higher in patients with C282Y homozygosity.

The findings of this study suggest that systemic iron overload does not cause OA directly but acts as a susceptibility factor. The systemic and synovial iron overload both contribute to increase the catabolic response of the articular cartilage to mechanical

stress, accelerating the progression of the osteoarthritic disease process. Our findings also suggests that the prevalence of musculoskeletal complications of HH is related to the magnitude of the iron overload, since there was a greater prevalence of OA in the homozygotes for the C282Y mutation, a genotype associated with higher systemic iron overload.

Resumo

A osteoartrose (OA) é a patologia articular mais frequente nos humanos. Afecta a articulação como um todo e as suas características principais são a destruição progressiva da superfície articular, a remodelação anormal do osso subcondral, a formação de osteofitos, o enfraquecimento das estruturas ligamentares, a atrofia muscular e, em alguns casos, a inflamação da membrana sinovial. Todas estas alterações levam a uma articulação dolorosa e incapaz de cumprir a sua função. Existem vários factores que aumentam o risco de vir a desenvolver OA mas a sequência exacta de acontecimentos que levam à destruição de uma articulação ainda não está totalmente esclarecida.

A Hemocromatose Hereditária (HH) é uma doença causada por mutações no gene HFE e caracteriza-se por causar uma sobrecarga sistémica de ferro e acumulação tóxica de ferro nas células parenquimatosas do fígado, do coração e das glândulas endócrinas. Está também associada a uma maior prevalência de patologias do aparelho osteoarticular, nomeadamente OA. Ainda não está definido qual o papel que a sobrecarga de ferro desempenha na génese da OA secundária à HH.

Para melhor entender os mecanismos moleculares responsáveis pelo aparecimento da OA secundária à HH, no decorrer do presente trabalho, foi induzida cirurgicamente OA no joelho de um modelo murino de hemocromatose e foram estudadas as alterações verificadas ao nível da cartilagem articular e do osso. Para além disso, para perceber se as diferentes mutações no gene HFE influenciavam a sobrecarga sistémica de ferro e as complicações osteoarticulares desta doença, foi estudada a prevalência de patologia osteoarticular em coortes de doentes com diferentes genótipos de HH.

Os ratinhos Hfe-KO apresentaram uma sobrecarga sistémica de ferro e uma deposição aumentada de ferro na membrana sinovial do joelho após a cirurgia. Às 8 semanas após cirurgia os ratinhos Hfe-KO apresentavam uma pontuação histológica da OA significativamente superior aos seus controlos saudáveis, traduzindo-se numa maior degeneração da cartilagem articular. Utilizando um aparelho de microtomografia computorizada foi possível estudar as alterações do osso subcondral ao nível da tíbia proximal. Esta apresentava-se com um volume ósseo aumentado e as trabéculas que a constituíam eram mais espessas do que as do grupo de controlo. Ao nível da expressão genética e imunohistoquímica observámos nos joelhos dos ratinhos Hfe-KO, um aumento significativo da expressão da metaloproteinase da matriz 3.

No estudo das coortes de doentes com HH, a maioria dos doentes eram homozigóticos para a mutação C282Y. A concentração de ferritina sérica e a saturação da trans-

ferrina sérica na altura do diagnóstico eram significativamente mais altas no grupo dos doentes homozigóticos quando comparadas com a dos doentes heterozigóticos compostos (C282Y/H63D). Para além disso os doentes homozigóticos para a mutação C282Y referiam uma maior prevalência de complicações osteoarticulares.

Os resultados deste estudo sugerem que a sobrecarga sistémica de ferro não é uma causa direta de OA mas sim um factor que aumenta a vulnerabilidade das articulações à sobrecarga mecânica. Supõe-se que a sobrecarga de ferro a nível sistémico e sinovial aumenta a resposta catabólica da cartilagem articular ao stress mecânico, acelerando o processo patológico da osteoartrose. Os dados obtidos sugerem ainda que a prevalência de complicações osteoarticulares na HH está relacionada com a magnitude da sobrecarga de ferro, uma vez que observámos uma maior prevalência de OA nos doentes homozigotos para a mutação C282Y, um genótipo associado a uma maior sobrecarga sistémica de ferro.

List of Papers

The thesis is based on the following papers:

1. Camacho A, Simão M, Ea H-K, Cohen-Solal M, Richette P, Branco J, Cancela ML, Iron overload in a murine model of hereditary hemochromatosis is associated with accelerated progression of osteoarthritis under mechanical stress. *Osteoarthritis and Cartilage* (2015), doi: 10.1016/ j.joca.2015.09.007.

2. Camacho A, Funck-Brentano T, Simão M, Cancela L, Ottaviani S, Cohen-Solal M, Richette P (2015) Effect of C282Y Genotype on Self-Reported Musculoskeletal Complications in Hereditary Hemochromatosis. *PLoS ONE* 10(3): e0122817. doi:10.1371/journal.pone.0122817

Chapter 1

Introduction

Osteoarthritis (OA) is the most common joint disease in humans (Glyn-Jones *et al.*, 2015). It affects the joint as a whole and is characterized by progressive articular cartilage destruction, abnormal subchondral bone remodelling, formation of osteophytes, ligament and periarticular muscles weakening (Arden & Nevitt, 2006) and in some cases synovial inflammation (Sellam & Berenbaum, 2010) that ultimately lead to a painful and impaired joint.

1.1 Prevalence and incidence of osteoarthritis

Prevalence

Systematic autopsy studies report that cartilage lesions, subchondral sclerosis and osteophytes are present in the knees of 60% of men and 70% of women aged over 70 (Arden & Nevitt, 2006).

Large-scale population health surveys, measuring either radiographic or self-reported OA (Table 1.1) provide current information about the prevalence of this disease. There is considerable variation among studies, which may be due to different disease definitions, anatomical locations and characteristics of the population sample such as age, genetic background (Lawrence *et al.*, 2008), occupation (O'Reilly *et al.*, 2000) or environmental exposures to toxins (Sun *et al.*, 2012).

OA prevalence increases indefinitely with age, and it is estimated that up to 8% of adults aged 25 and older in the United States of America have clinical OA of some joint (Lawrence *et al.*, 2008). Worldwide OA affects 9.6% of men and 18% of women aged >60 years (Woolf & Pfleger, 2003). It is the sixth leading cause of disability-adjusted life years (DALYs) accounting for 3% of the total global DALYs (Woolf & Pfleger, 2003) and it is projected that in high-income countries this figure will remain similar until 2030 (Mathers & Loncar, 2006).

Table 1.1: Prevalence of osteoarthritis in large-scale surveys. National Health and Nutrition Examination Survey (NHANES);

Source	Age	Population	Site	Criteria	Overall %
(Dillon *et al.*, 2006)	>60	NHANES-III, USA	Knee	Radiographic	37.4%
				Symptomatic	12.1%
(Felson *et al.*, 1987)	>63	Framingham, USA	Knee	Radiographic	33 %
				Symptomatic	9.5%
(Kim *et al.*, 2014)	>50	Framingham, USA	Hip	Radiographic	18.5%
				Symptomatic	4.0%
(Jordan *et al.*, 2007)	>45	Johnston County, USA	Knee	Radiographic	28%
				Symptomatic	16%
(Jordan *et al.*, 2009)	>45	Johnston County, USA	Hip	Symptomatic	10%
				Radiographic	28%
(van Saase *et al.*, 1989)	>45	Zoetermeer, Netherlands	Knee	Radiographic	19.2%
			Hip	Radiographic	8.1%
(Dahaghin *et al.*, 2005)	>55	Rotterdam, Netherlands	Hand	Radiographic	28.3%
				Hand pain	16.8%
(Vavken & Dorotka, 2011)	>15	Austria	Entire body	Self-reported	18.8%

Incidence

The incidence of OA depends on the disease process that initiated the cartilage degeneration. Since it is impossible to know for certain the onset of the disease process, most of the studies base their results on radiographic criteria to assess the incidence and progression of OA, giving a skewed estimate of the true incidence of this disease. Also most of the incidence rates reported reflect a mix of primary and secondary OA.

Estimates from the Australian population suggest that osteoarthritis affects women more frequently than men across all age groups (2.9 per 1000 vs. 1.7 per 1000) (Mathers *et al.*, 1999), findings corroborated by other authors in a meta-analysis (Srikanth *et al.*, 2005). The incidence of radiographic disease is higher than of symptomatic OA (Felson *et al.*, 1995) but both increase with age, approaching 1% per year for symptomatic knee OA in elderly females (Oliveria *et al.*, 1995) and 2.5% per year for radiographic knee OA in individuals of both sexes aged >55 years (Cooper *et al.*, 2000).

1.2 Causes of osteoarthritis

Usually, OA is classified as primary (idiopathic) or secondary, according to the mechanism that initiates the cartilage degradation (Ferri, 2015).

Primary OA is considered a process that occurs with ageing and normal usage of the joint. However, some authors defend that this classification is not valid, and that all OA is secondary (Brandt *et al.*, 2009a), since the degenerative process derives from either an abnormal joint structure or an abnormal distribution of force across the different joint tissues (Murray, 1965; Solomon, 1976; Mitchell & Cruess, 1977). In this context, primary OA could be classified as a case of normal forces acting on an abnormal joint structure.

Indeed, the ageing process leads to chondrocyte senescence undermining their ability to maintain the cartilage matrix (Martin *et al.*, 2004) and causing a decrease in matrix proteoglycan content. This is accompanied by a decrease in water content, altering the force-absorbing characteristics of the cartilage, making it more susceptible to degeneration from normal joint use (Buckwalter *et al.*, 2005).

Several disorders cause direct or indirect damage to articular cartilage leading to joint degeneration (Buckwalter & Mankin, 1998). A brief summary of these conditions is presented in Table 1.2. The presumed mechanisms leading to OA development are either damage to articular cartilage or alterations to joint alignment, congruity or stability that eventually lead to joint degeneration.

Since the focus of this dissertation is the role of iron overload in the development of OA, the relationship between Hereditary Hemochromatosis (HH) and OA will be described in more detail.

Table 1.2: Causes of secondary OA and their presumed initiating mechanism

Cause	Presumed Mechanism
Acute trauma (Intra-articular fracture, joint surgery)	Damage to articular cartilage or incongruity of joint or both
Chronic joint overload (sports, occupational)	Damage to articular cartilage or subchondral bone or joint incongruity
Hemochromatosis	Mechanism unknown
Inflammatory arthritis	Synovial membrane inflammation induces bone, ligament and cartilage destruction
Ochronosis	Deposition of homogentisic acid polymers in articular cartilage
Gaucher's disease	Bone necrosis or pathological fracture leads to incongruity of joint
Dysplasia of joint and cartilage (developmental or congenital)	Abnormal shape of the joint or abnormal articular cartilage or both
Acromegaly	Overgrowth of cartilage produces joint incongruity
Calcium pyrophosphate deposition disease	Accumulation of crystals in articular cartilage
Infection of joint	Destruction of articular cartilage

Table 1.2: Causes of secondary OA, cont.

Cause	Presumed Mechanism
Neuropathic arthropathy (Charcot joints due to diabetes mellitus, syphilis, syringomyelia, myelomeningocele, amyloidosis)	Loss of proprioception and joint sensation
Avascular necrosis	Bone necrosis leads to collapse of the articular surface and incongruity of the joint
Hemophilia	Recurrent hemarthroses leads to proliferative synovitis and cartilage degradation
Ligament injuries	Instability of the joint

1.3 Hemochromatosis

1.3.1 Characteristics and molecular causes

There are at least five recognised subtypes of hemochromatosis (Table 1.3), each with distinct genetic and molecular profiles (Kanwar & Kowdley, 2013). These can be grouped into those associated to hemochromatosis (HFE) gene mutations and those independent of HFE gene mutations, with the latter being more relevant in the Asia-Pacific populations. We will focus on the Type I, or classical hemochromatosis, resulting from the mutation of the HFE gene, since it is the most common type on the European population.

Hereditary hemochromatosis (HH) (Figure 1.1) is an autosomal recessive disorder characterized by increased absorption of dietary iron, and rapid iron release from intracellular storage sites, which leads to abnormal accumulation of iron in several organs, particularly in the liver, heart, endocrine organs and joints (Allen *et al.*, 2008; Guggenbuhl *et al.*, 2011b). Large-scale screening studies in populations of Northern European descent have estimated that the prevalence of the disease is 0.5 to 11.5 per 1,000 persons and that it affects predominantly males (Tanner *et al.*, 1985; Karlsson *et al.*, 1988; Edwards *et al.*, 1988; Leggett *et al.*, 1990; Phatak *et al.*, 1998; Asberg *et al.*, 2001; Steinberg *et al.*, 2001), making it one of the most common genetic disorders among caucasians.

Trousseau (Trousseau, 1865) originally reported the triad of cirrhosis, diabetes and skin tanning, and Von Recklinghausen completed the description, reporting the presence of strong iron deposits within the liver of those patients, suggesting that iron played a central role in the development of the disease (Von Recklinghausen, 1889).

The research around this disease continued, but it was only in 1977 that Simon et al. were able to link it to a region in chromosome 6, close to the human leukocyte antigen (HLA) genes (Simon *et al.*, 1977).

More recently the role of genetic factors was elucidated when Feder (Feder *et al.*, 1996) identified two missense alterations: cysteine-to-tyrosine substitution at amino acid 282 (C282Y) and histidine-to-aspartic acid substitution at position 63 (H63D), in an major histocompatibility complex (MHC) class I-like gene in 83% of their HH patients.

The gene was then named HFE and follow-up studies showed that these mutations

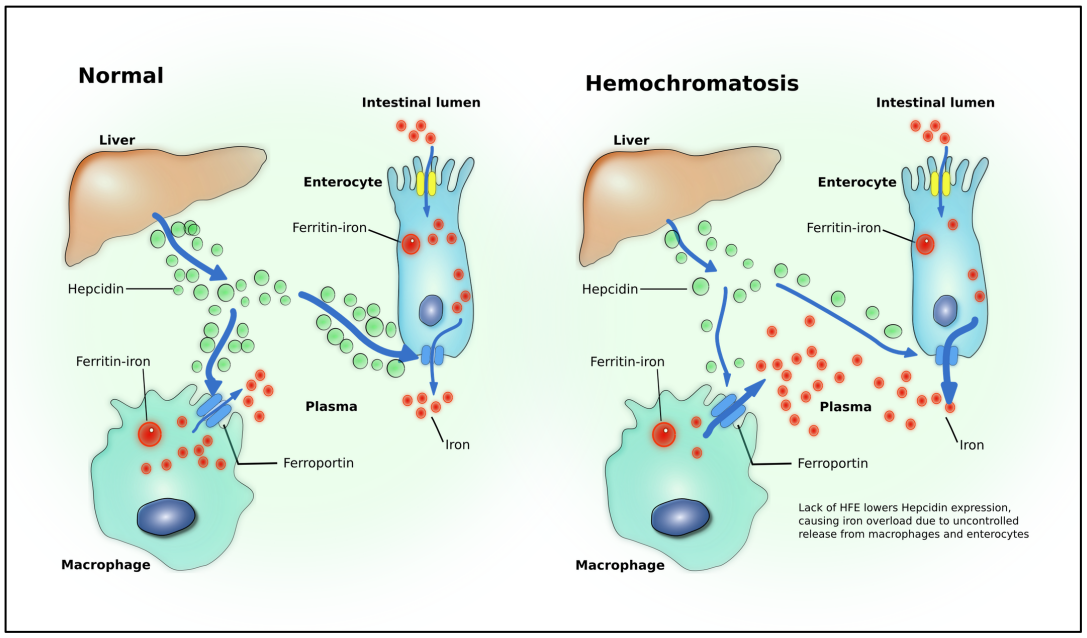

Figure 1.1: Iron metabolism in healthy patients and in hemochromatosis. Adapted from P. C. Adams and J. C. Barton. Haemochromatosis. Lancet, 370(9602):1855–60, Dec 2007.

affected the ability of the HFE protein to interact with the transferrin receptor (Waheed *et al.*, 1997; Feder *et al.*, 1998), thus failing to form the membrane-associated iron-sensing complex composed of HFE, transferrin receptor 2 (TFR2) and hemojuvelin (HJV). That, in turn, leads to low levels of hepcidin antimicrobial peptide (HAMP) transcription (Bridle *et al.*, 2003; Giannetti & Björkman, 2004; Waheed *et al.*, 2008; Schmidt *et al.*, 2008; D'Alessio *et al.*, 2012).

HAMP is the main regulator of iron homeostasis (Viatte & Vaulont, 2009). It is a peptide produced by the liver in response to anaemia, hypoxia and inflammation (Nicolas *et al.*, 2002) and acts by inducing the degradation of the iron-exporter protein ferroportin (FPN) (Nemeth *et al.*, 2004). The HAMP mediated internalization and degradation of FPN causes intracellular iron retention in enterocytes, macrophages and hepatocytes, lowering blood serum iron levels (Pantopoulos *et al.*, 2012). Inversely the lack of HAMP causes a release of iron from intracellular storage and consequent systemic iron overload and clinical disease (Ganz, 2013).

Table 1.3: Types of hereditary hemochromatosis, adapted from D. Ekanayake et al. , Recent advances in hemochromatosis: a 2015 update, *Hepatol Int*, Apr 2015

Type	Gene	Function	Prevalence	Associated features
Type I	HFE	Upregulates hepcidin	Most common form worldwide; accounts for 90% of cases	Classical hemochromatosis
Type IIA	HJV	Upregulates hepcidin	Rare; more common than type IIB	Severe early onset
Type IIB	HAMP	Inhibits iron uptake by enterocyte	Rare	
Type III	TFR2	Hepatic transferrin receptor, possible role in hepcidin regulation	Rare in Europe. Most common form in Japan. Present in Italy and Brazil.	Can have juvenile or adult onset. Most cases are adult and have a more severe course than type I.
Type IV	FPN	Iron export	Rare	Reduced end-organ damage

1.3.2 Clinical presentation

Classical hemochromatosis usually manifests itself in middle-aged patients and the signs can range from simple biochemical abnormalities such as elevated serum ferritin to severe organ damage and disease (Pietrangelo, 2010). The variations in signs and symptoms occur because the HFE mutations only predispose the individual to hemochromatosis; additional factors such as simultaneous mutations in other genes, gender, alcohol intake, obesity and concomitant liver disease are required (Olynyk *et al.*, 1999). Some organs such as heart, liver, pancreas, pituitary gland and joints, are more readily affected by the iron overload (Bacon & Sadiq, 1997). The classic presentation of liver cirrhosis, bronze-colored skin, diabetes, heart disease and joint inflammation is rare nowadays, due to the increased awareness for the disease and better screening programs (Ekanayake *et al.*, 2015). Most of the patients nowadays present nonspecific symptoms such as weakness, lethargy, arthralgia, and also unspecific signs such as hepatomegaly. The transferrin saturation is frequently elevated and, in later stages of the disease, the serum ferritin also increases, indicating iron deposition in soft tissues (Pietrangelo, 2010). Patients with serum ferritin levels $>1000\mu g\,l^{-1}$ at diagnosis have an increased risk of cirrhosis and death (Barton *et al.*, 2012).

1.3.3 Diagnosis

Diagnosis is made by a combination of clinical features, laboratory tests, imaging and genetic testing. Universal screening for HH is not recommended but, if a patient is

symptomatic, has hyperferritinemia or a first degree relative with hemochromatosis, determination of transferrin saturation and of serum ferritin levels is indicated (Crownover & Covey, 2013).

After ruling out other causes that affect body iron levels and other causes of liver damage such as viral and alcoholic hepatitis, a transferrin saturation >45% and ferritin levels >300µg l^{-1} in men or >200µg l^{-1} in women, is a strong indication for HFE genetic screening (Crownover & Covey, 2013). Homozygotes for C282Y mutation with elevated iron parameters do not need a confirmatory biopsy and can start treatment. Patients with other HFE genotypes should undergo a liver biopsy to assess the need for treatment.

Liver biopsy is the best determinant of fibrosis and cirrhosis and it has a great value in determining patient prognosis. An hepatic iron concentration (HIC) >4000 µg is diagnostic of HH phenotype and is a formal indication to begin treatment with phlebotomy (Kanwar & Kowdley, 2014).

With the advent of less invasive methods for measuring HIC such as T2* magnetic resonance imaging (MRI) (St Pierre *et al.*, 2005) and for staging liver disease such as transient elastography (Adhoute *et al.*, 2008), the necessity to perform a liver biopsy is diminishing.

1.3.4 Treatment

Once the disease is diagnosed a treatment or surveillance protocol is initiated. For patients with serum ferritin in the normal range a yearly follow-up with transferrin saturation and serum ferritin measurement suffices (European Association For The Study Of The Liver, 2010).

If the ferritin levels are elevated, treatment with phlebotomy, in order to bring serum ferritin to a level between 50 µg l^{-1} and 100 µg l^{-1}, is indicated (European Association For The Study Of The Liver, 2010).

Phlebotomy is the only widely accepted treatment. It works by directly reducing the haemoglobin stores of iron and by inducing erythropoiesis, which mobilises stored iron (Ekanayake *et al.*, 2015). Even though no randomised trial documented the efficacy of this treatment, it is known that it has beneficial effects in some symptoms of the disease. Treatment with iron chelators or with erythrocytapheresis have been described in the literature (Kanwar & Kowdley, 2014).

Liver transplantation is a curative treatment option, and post-transplant outcomes are comparable with other diagnoses. However, when compared to phlebotomy, it has a much higher cost and entails chronic immunosuppression (Bardou-Jacquet *et al.*, 2014).

1.3.5 Hemochromatosis and arthropathy

The first description of HH-related arthropathy is attributed to Schumacher, who reported the cases of two patients with an early-onset arthritis closely associated with the other symptoms of hemochromatosis, and with greater involvement of proximal interphalangeal and metacarpal-phalangeal joints (Schumacher, 1964). These patients also had hemosiderin in the synovial lining cells, without proliferative or inflammatory changes.

At the time Schumacher speculated that iron could act as a toxin causing physical or metabolic alterations in the cartilage.

Since the original description, several studies have focused on the prevalence of musculoskeletal complications in hemochromatosis, reporting (*i*) an increased prevalence of joint pain and arthritis (McDonnell *et al.*, 1999; Richette *et al.*, 2010; Sahinbegovic *et al.*, 2010b) and (*ii*)increased rates of joint replacement surgery (Sahinbegovic *et al.*, 2010a; Wang *et al.*, 2012; Elmberg *et al.*, 2013) in patients with mutations of the HFE gene. However, the mechanism by which iron overload damages the joints in HH is still undefined.

Histological studies of synovial tissue in HH-related arthritis show superficial deposits of hemosiderin, minor proliferation of synovial cells, and less inflammatory cell infiltrate when compared to rheumatoid arthritis (Muirden & Senator, 1968), but more macrophages and neutrophils when compared to primary OA (Heiland *et al.*, 2010). The latter study suggests that the increased accumulation of neutrophils may lead to production of matrix-degrading enzymes causing cartilage degradation. Another study (Carroll *et al.*, 2010) found a two to threefold increased ferritin concentration in the synovial fluid of OA patients who were heterozygous for HFE gene mutations, compared to patients without any HFE mutation, but they found no differences in the concentration of selected inflammatory cytokines or matrix metallopeptidase 3 (MMP3) between the two groups.

Patients with HH were found to have increased non-transferrin bound iron (NTBI) concentrations in serum (de Valk *et al.*, 2000). This potentially toxic iron form could also be present in excess in the joints of HH patients and cause cellular damage to synovial membrane and chondrocytes due to its propensity for generating reactive oxygen species (ROS) (Brissot *et al.*, 2012).

In conclusion, while significant progress has been made in understanding the molecular mechanisms of iron overload in HH the pathogenesis of HH-related OA is still poorly understood (Husar-Memmer *et al.*, 2014).

1.4 Pathogenesis of osteoarthritis and tissues affected

Osteoarthritis is the pathophysiological response of a synovial joint to insult, either mechanical, metabolic or inflammatory (Brandt *et al.*, 2009b). It is a disease of the joint as a whole, meaning that lesions in either cartilage, bone, ligaments, synovial membrane or periarticular muscles can ultimately lead to the destruction of the extracellular matrix of articular cartilage, subchondral bone sclerosis, osteophyte formation, joint effusion and loss of function (Figure 1.2).

The chondrocytes are responsible for the synthesis and maintenance of the articular extracellular matrix (ECM) (Muir, 1995). Their metabolism, and subsequent articular cartilage growth and function, is influenced by mechanical stimuli (Lee *et al.*, 2000; Bougault *et al.*, 2012) and by mediators from the synovial membrane (Pelletier *et al.*, 1985) reinforcing the notion that OA is not only a disease of the articular cartilage but of the whole joint.

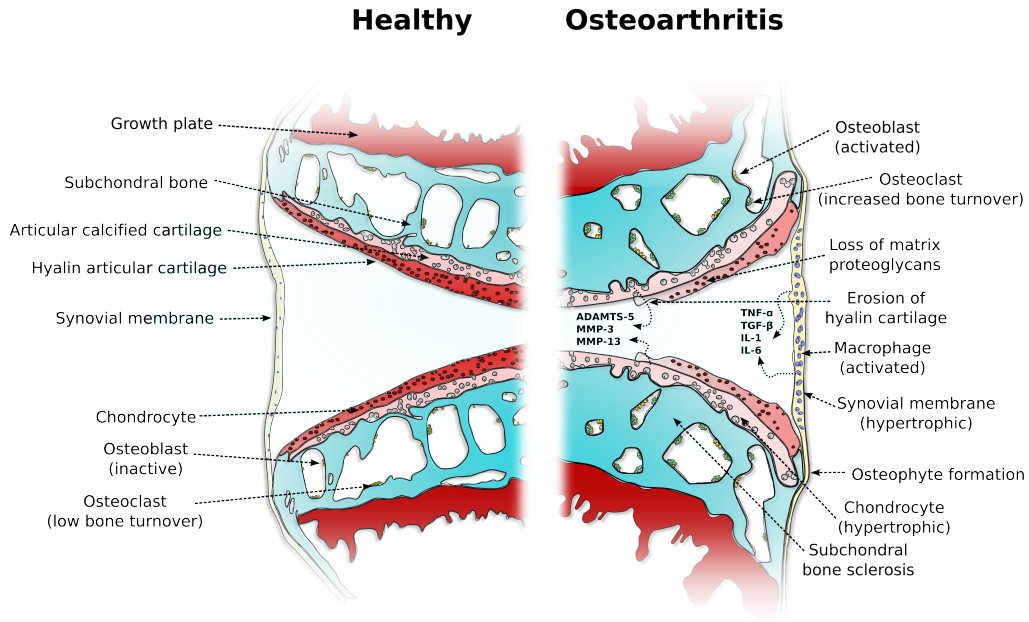

Figure 1.2: Structural changes and selected signalling and effector molecules in the development of osteoarthritis. ADAMTS: a disintegrin and metalloproteinase with thrombospondin-like motifs; IL: interleukin; MMP: matrix mettaloproteinase; TGF: transforming growth factor; TNF: tumor necrosis factor.Adapted from S. Glyn-Jones et al.Osteoarthritis. Lancet, Mar 2015

Changes to cartilage

Articular hyaline cartilage is mainly constituted by a mesh of type II collagen fibres, stabilised by other collagen types and non-collagenous proteins, which provide shear and tensile strength to the cartilage. Hyaluronic acid and proteoglycans are embedded in this structure and they retain water in the cartilage providing compressive resistance (Glyn-Jones *et al.*, 2015). Water is the most abundant component of articular cartilage and most of it is contained within the intrafibrillar space of the matrix, held in place by the negative charge of the proteoglycans (Pearle *et al.*, 2005).

Chondrocytes, which are highly differentiated cells, have a limited capacity for proliferation or migration since they are encased in this dense extracellular matrix. In the event of a lesion, they are unable to reach the damaged areas and the matrix components that they would produce cannot fill the extracellular matrix (ECM) defects (Buckwalter *et al.*, 2005). Although chondrocytes synthesise sufficient macromolecules to maintain the structure of the ECM, their capacity to further increase the synthesis of proteoglycans or collagen is limited and insufficient to repair a significant tissue defect (Muir, 1995).

Another obstacle to the repair of articular lesions is that adult articular cartilage is a

dense and avascular tissue. Therefore disruption of the tissue does not cause fibrin clot formation or migration of undifferentiated cells to the site of tissue damage, where they could then proliferate, differentiate and synthesise a new matrix.

The progression of the cartilage damage occurs in three overlapping phases: damage to the chondrocytes or to the extracellular matrix, chondrocyte response to the insult, and the decline of chondrocyte anabolic processes and eventual loss of cartilage tissue. The initial damage can result from several causes (Table 1.2), but most frequently results from a mechanical insult.

The damage to the collagen network, which usually begins in the superficial layer of the cartilage, is accompanied by loss of the articular cartilage proteoglycans (Radin *et al.*, 1973; Squires *et al.*, 2003). This disrupts the extracellular matrix framework, increasing the permeability and decreasing the tensile strength of the tissue (Bank *et al.*, 2000), allowing the proteoglycans to expand and increasing the water concentration of the extracellular matrix (Inerot *et al.*, 1978). These alterations of the cartilage are clinically referred to as chondromalacia, and are accompanied by fibrillation of the surface layer, predisposing the remaining cartilage to further mechanical insult (Setton *et al.*, 1993). These high-strain mechanical stimuli that act on the chondrocytes are transduced by mechanosensitive ion channels leading to biochemical-metabolic responses (Lee *et al.*, 2014).

One of the responses to mechanical stress is increased production of nitric oxide (NO), an inflammatory mediator, (Fermor *et al.*, 2002) which increases apoptosis (Blanco *et al.*, 1995), and leads to a decrease in chondrocytes proteoglycan production, replicative capacity and telomere length (Yudoh *et al.*, 2005). Mechanical insult also induces the expression of genes encoding for several collagenases (metallopeptidase 1, 3 and 13), and aggrecan-degrading enzymes(ADAMTS 4 and 5) (Lee *et al.*, 2005; Burleigh *et al.*, 2012), resulting in degradation of the collagen network and of the proteoglycan structure, further weakening the mechanical properties of articular cartilage. There is a small repair component associated with this stage of OA, with an increase in proteoglycan (Venn *et al.*, 1995) and type II collagen (Hermansson *et al.*, 2004) synthesis, and chondrocyte proliferation (De Ceuninck *et al.*, 2001). If the insult to the cartilage is maintained the cartilage damage will progress since newly synthesised proteoglycans will fail to aggregate (Moskowitz *et al.*, 1979) and the type II collagen will not be incorporated in the existing collagen network (Buckwalter *et al.*, 2005).

The final stage of OA occurs when cartilage is unable to recover from the mechanical and chemical insults, resulting in chondrocyte hypertrophy, calcification of the ECM, and complete destruction of the articular cartilage (van der Kraan & van den Berg, 2012). With ageing, the chondrocytes are less responsive to anabolic growth factors, and synthesise smaller and less functional proteoglycans (Martin & Buckwalter, 2003). In addition there is an accumulation of advanced glycation end-products that affect the mechanical properties of the ECM (Shane Anderson & Loeser, 2010), thus OA is more commonly seen in the elderly.

Changes to subchondral bone

Articular cartilage is seated on a subchondral plate, a thin layer of cortical bone supported by the trabecular bone of the metaphysis. Articular cartilage is too thin to effectively absorb the shock of impulsive loads. The bone and soft tissues attenuate these forces far better (Radin & Paul, 1970).

The subchondral bone acts as a cushion to dampen the forces acting upon the articular cartilage, and the probable mechanism for shock absorption is limited trabecular fracture (Simon *et al.*, 1972). This would result in the formation of a fracture callus and increased remodelling of the subchondral bone (Radin & Rose, 1986; Hayami *et al.*, 2004).

The remodelling process caused by repetitive and unattenuated impulsive loads leads to increased trabecular thickness and reduced size and number of the intertrabecular spaces, resulting in sclerosis and stiffening of the subchondral plate (Radin *et al.*, 1991). With MRI imaging it is possible to detect bone-marrow lesions related to trabecular microfractures at different stages of healing that are localized in the areas with most cartilage damage (Taljanovic *et al.*, 2008). Some authors (Imai *et al.*, 1989; Brandt *et al.*, 2009b) propose that the rigid subchondral bone increases the shear stress at the cartilage/bone interface, causing splitting between the layers of bone and cartilage which results in cartilage degeneration. Conversely, loss of cartilage integrity could lead to increased loading of the subchondral bone and subsequent remodelling (Brandt *et al.*, 2009b). The exact sequence of events is still undefined. Features of endochondral ossification such as chondrocyte hypertrophy, cartilage degradation and vascular invasion are replayed in osteoarthritis, resulting in tidemark advances (Oegema *et al.*, 1997) and osteophyte formation (Moskowitz & Goldberg, 1987; Hashimoto *et al.*, 2002). These osteophytes can restrict joint motion and be a source of pain at the limits of joint motion.

Changes to periarticular tissues

The cells in the synovial membrane produce the synovial fluid, which contributes to cartilage nourishment, and lubricates and protects the articular surfaces. Synovial fluid includes lubricants such as hyaluronic acid (HA) (Smith & Ghosh, 1987) and lubricin (Jay *et al.*, 2007), but in patients with OA the lubrication capability of the synovial fluid is diminished (Ludwig *et al.*, 2012), possibly owing to lower concentration and lower molecular mass of the HA (Moreland, 2003).

The cartilage and subchondral bone degradation products found on OA act on the synovial membrane, leading to a persistent low grade synovitis (Brandt *et al.*, 2009a), and resulting in the production of proinflammatory cytokines, such as interleukin 1 (IL1) and tumour necrosis factor α (TNF-α) (Smith *et al.*, 1997). These cytokines are released into the synovial fluid and act on the chondrocytes by inducing the biosynthesis of NO (Stadler *et al.*, 1991). In turn this results in reduced proteoglycan synthesis (Taskiran *et al.*, 1994), reduced type II collagen synthesis (Goldring *et al.*, 1988), and activation of metalloprotease activity (Murrell *et al.*, 1995), with the end result being damage to the articular cartilage. In the late stages of OA there is also a contracture of the articular capsule and ligaments resulting in a decreased range of motion. Periarticular

muscle atrophy can also be seen in late-stage OA, since joint pain leads to decreased mobilization causing secondary muscular atrophy.

1.5 Risk factors for osteoarthritis

Mechanical overload of the joint not only literally crushes the articular cartilage and subchondral bone (Brandt *et al.*, 2009b),but also leads to the expression of proteases that cause ECM degradation (Burleigh *et al.*, 2012). Not all joints subjected to mechanical overload develop OA. Follow-up studies of patients with anterior cruciate ligament or meniscus tears, report that 20 years after the diagnosis, the prevalence of OA was on average 50% (Lohmander *et al.*, 2007). This was also observed among subjects with paediatric orthopaedic hip conditions in whom the prevalence of hip OA 30 years after the diagnosis is 60-70% (Weinstein, 2000).

Although this does not mean that the patients which did not develop OA will not develop it in the future, it reinforces the hypothesis that there are other factors that are likely to contribute to the development of OA, namely environment (Hunter *et al.*, 2002), gender (Yoshimura *et al.*, 2009), diet (La Grange *et al.*, 2001) and genetics (Tsezou, 2014).

From the biomechanical point of view several factors can cause mechanical overload of the joint, with the most common being joint incongruity, joint instability, loss of limb alignment, diminished muscle strength or excess body weight. Each of these will now be briefly described.

Joint incongruity

Joint incongruity may arise from intra-articular fractures (Marsh *et al.*, 2002; Murray *et al.*, 2004); from congenital abnormalities such as hip dysplasia (Jacobsen & Sonne-Holm, 2005); from osteonecrosis of the femoral head (Ohzono *et al.*, 1991), humeral head (Hasan & Romeo, 2002) or femoral condyles (Lotke & Ecker, 1988); from meniscectomy (Roos *et al.*, 1998) or from altered geometry of the metaphysis such as femoroacetabular impingement (Ganz *et al.*, 2003), among other causes. It results in abnormal load distribution within the joint and is associated with a very high risk of developing osteoarthritis of the affected joint.

Joint instability

Experimental data has shown that joint instability causes an abnormal distribution of forces across the articular cartilage (Papageorgiou *et al.*, 2001), leading to extracellular matrix lesions and subsequent cartilage degeneration (Kamekura *et al.*, 2005).

Traumatic injuries that cause ligamentous injury can also cause intra-articular bleeding, meniscal or osteochondral lesions and damage the articular capsule and peri-articular muscles. These associated lesions may contribute to OA development in unstable joints (Roos, 2005; Øiestad *et al.*, 2009). Clinical studies on the natural history of joint instability show an increased incidence of knee OA in patients with anterior cruciate ligament insufficiency (Kannus & Järvinen, 1989; Hill *et al.*, 2005), of shoulder OA in patients with

massive rotator cuff tears (Neer *et al.*, 1983) or traumatic anterior instability (Buscayret *et al.*, 2004) and of ankle OA in patients with traumatic ligament injury (Valderrabano *et al.*, 2006).

Limb alignment

In the lower limb, the ground reaction force is transmitted across a linear axis extending from the centre of the hip to the centre of the talus, which is often referred to as the load-bearing axis (LBA). The hip-knee-ankle (HKA) angle describes how closely the mechanical axes of the femur and tibia are lined up with each other. Perfect alignment would have the femoral and tibial long axes in line with each other. Surveys show that in healthy adults the HKA is 1º varus (Cooke *et al.*, 1997) positioning the knee centre marginally lateral to the LBA, causing 60-70% of the ground reaction force to be transmitted through the medial compartment (Andriacchi, 1994). The increased compressive stress is associated with increased incidence and progression of knee OA (Sharma *et al.*, 2010), and this could explain the greater prevalence of medial compartment involvement in OA(McAlindon *et al.*, 1992).

Muscle strength

The quadriceps is the main muscle of the anterior compartment of the thigh and acts as a brake, decelerating the descent of the leg during the swing phase of gait. Quadriceps weakness (Slemenda *et al.*, 1997) and atrophy (Fink *et al.*, 2007) are common clinical findings in patients with knee OA, but little is known about why it develops.

One hypothesis is that diseased joints, due to altered proprioception or pain, transmit afferent inputs that inhibit the motor neuron stimulation (Rutherford *et al.*, 1986) causing a diminished quadriceps muscle activation (Lewek *et al.*, 2004). This leads to altered movements when walking and to different patterns of muscle activation in the lower limb, particulary an imbalance between the quadriceps and the hamstring (Hortobágyi *et al.*, 2005), which may interfere with the joint's ability to dissipate the forces arising from contact with the ground, ultimately contributing to OA progression (Childs *et al.*, 2004).

Individuals with hip OA also exhibit a decrease in the cross-sectional areas and strength of pelvic and thigh muscles, when compared to healthy age-matched controls (Arokoski *et al.*, 2002) making it clear that alterations in muscle function are not limited to the knee. Muscle strength is also associated with the initiation of hand OA, but it seems to have an inversed effect, in that, men with high grip strength are at increased risk for developing hand OA (Chaisson *et al.*, 1999).

Age and gender

Age is the biggest risk factor for the development of OA (van Saase *et al.*, 1989; Felson *et al.*, 2000; Lawrence *et al.*, 2008). While the exact mechanism is not clear, it could reflect an accumulation of biomechanical insults, senescence of articular chondrocytes

with corresponding decline in function (Martin *et al.*, 2004), or a reduced capacity to respond to biomechanical stresses owing to age-related decrease in neuromuscular joint protective mechanisms (Mau-Moeller *et al.*, 2013; Maden-Wilkinson *et al.*, 2014), or a combination of all these factors. Osteoarthritis is more common in women than in men (Lawrence *et al.*, 2008). The role of oestrogen in the development and symptoms of OA in humans is still undefined (Nevitt *et al.*, 2001), but a recent study found evidence that in mice, testosterone accelerated the progression of surgically induced OA (Ma *et al.*, 2007) suggesting that chondrocyte metabolism may be influenced by hormones.

Genetic background

Osteoarthritis seems to have a genetic basis, as pointed out by genome-wide association studies which have found several genes to be differentially expressed between cases and controls, namely up-regulation of the apoptosis pathway (Ramos *et al.*, 2014), up-regulation of the Wnt pathway (Velasco *et al.*, 2010) and down-regulation of oxidative defence genes (Aigner *et al.*, 2006). These large scale genome-wide studies also identified several loci associated with increased OA susceptibility (Kerkhof *et al.*, 2010; Demirkan *et al.*, 2012; Evangelou *et al.*, 2014), but the molecular mechanisms by which each of these genetic variations increase OA risk are still not clear (Tsezou, 2014).

Bone mineral density

Increased bone mineral density (BMD) has been identified as a potential risk factor for hip and knee OA in several epidemiological studies (Hannan *et al.*, 1993; Burger *et al.*, 1996; Hochberg *et al.*, 2004; Hardcastle *et al.*, 2015). While the association between radiographically diagnosed OA and increased BMD is widely accepted, it is possible that confounding factors such as activity levels or bone size may explain this association (Javaid & Arden, 2013). In addition, increased BMD is more strongly related to the presence of osteophytes than to joint space narrowing (Nevitt *et al.*, 1995), suggesting that increase BMD may predispose to the bony features of OA (appearance of osteophytes and subchondral sclerosis) rather than directly to cartilage loss (Hardcastle *et al.*, 2014). Since most epidemiological studies use the Kellgren-Lawrence (KL) criteria, in which osteophytes are considered as a radiographic sign of OA (Schiphof *et al.*, 2008), the bony features of OA seen in individuals with increased BMD would lead to a diagnosis of radiographic OA, yet cartilage damage could in fact be minimal.

Obesity

Weight gain is strongly associated with increased progression of cartilage damage (Bucknor *et al.*, 2015), and obesity is a well-established risk factor for knee OA (Hochberg *et al.*, 1995; Oliveria *et al.*, 1999), although the effect could also depend on knee alignment (Felson *et al.*, 2004), but does not increase the risk of developing hip OA (Mork *et al.*, 2012). The primary mechanism for this association probably involves the mechanical overload of the knee joints during weight-bearing activities, causing damage to carti-

lage and ligaments. Quadriceps muscle strength seems to be maintained in the obese individuals at risk for knee OA (Segal *et al.*, 2011). Metabolic alterations associated with obesity, namely hyperglycaemia, raised triglycerides, hypertension or reduced high-density lipoproteins are also associated with an increased risk of OA (Monira Hussain *et al.*, 2014). Cartilage metabolism could also be altered in obese individuals, explaining the increased prevalence and incidence of OA in non-weight bearing joints, such as hand and wrist, in obese individuals (Carman *et al.*, 1994).

Diet

Oxidative damage to cartilage and periarticular tissues caused by reactive oxygen species could increase the susceptibility to OA (Afonso *et al.*, 2007), so an increased intake of antioxidants might be associated with a lower rate of OA (McAlindon *et al.*, 1996a). Longitudinal population studies found a reduced progression rate of knee OA in individuals with a high reported intake of vitamin C (McAlindon *et al.*, 1996a; Peregoy & Wilder, 2011), but a more recent study found that individuals who were in the highest tertile of circulating vitamin C levels had an increased incidence of radiographic knee osteoarthritis, and similar results were found for circulating vitamin E levels (Chaganti *et al.*, 2014).

Vitamin D metabolites influence the development and maturation of calcifying cartilage (Schwartz *et al.*, 1989), and it has been postulated that low levels of vitamin D could increase the risk of incident hip and knee OA (McAlindon *et al.*, 1996b; Lane *et al.*, 1999), however a large longitudinal study (Felson *et al.*, 2007) and a randomized clinical trial(McAlindon *et al.*, 2013) both found that vitamin D supplementation failed to reduce knee pain or OA progression.

Kashin-Beck disease (KBD) is a condition that affects the chondrocytes of the articular cartilage and growth plate, leading to secondary deformities of the long bones and to cartilage degeneration (Kolsteren, 1992). While the aetiology is unknown, several studies show a relationship with selenium (Sun *et al.*, 2012; Zhang *et al.*, 2011) and iodine (Yao *et al.*, 2011) deficiencies, mycotoxins on grain (Liu *et al.*, 2014), and the presence of organic material in drinking water (La Grange *et al.*, 2001) reinforcing the importance of nutrients in the pathogenesis of OA.

Occupation and physical activity

Mechanical overload of joints caused by repetitive motions could lead to OA. Occupations such as construction work and mining, which require repetitive knee-bending and heavy lifting, have been associated with knee OA in a middle-aged working population (Anderson & Felson, 1988; O'Reilly *et al.*, 2000). Hand OA was also associated with occupations demanding increased manual dexterity, such as cleaning work, clothing industry works and masonry (Rossignol *et al.*, 2005).

Physical activity is potentially detrimental to the joint should it lead to repetitive overload, namely in individuals presenting with joint instability, abnormal limb alignment or sports-related injuries (Buckwalter & Lane, 1997). Moreover, there is an increased risk

of hip and knee OA among former elite athletes when compared to untrained controls (Marti *et al.*, 1989; Kujala *et al.*, 1994) and to non-elite athletes (Lindberg *et al.*, 1993; Roos *et al.*, 1994), but for most individuals practising recreational sports at a normal level there is no increased risk of developing OA (Lequesne *et al.*, 1997). It is still unclear if the increased prevalence of OA in elite athletes is related with high-intensity loading of the joints or if it is secondary to an increased prevalence of joint injuries (Hunter & Eckstein, 2009).

1.6 Diagnosis of osteoarthritis

Osteoarthritis is classically diagnosed by clinical examination and plain film radiographs. Patients in the early phases of OA usually have minimal signs and symptoms, but there may be discrete loss of motion at the extremes or soreness after excessive exercise that resolves with a few days of rest or anti-inflammatory medication. With the progression of the disease the symptoms can aggravate. There will be pain with ordinary daily activities, and physical examination will reveal joint pain, loss of motion, crepitus, deformity and joint effusion. By this stage the disease is probably irreversible. Symptoms fluctuate with time and are influenced by comorbid pathologies. Patients usually only seek medical attention when symptoms affect their daily lives, and the fluctuating course hinders the diagnosis of the disease in its early stages.

The features of osteoarthritis seen in plain radiographs include joint space narrowing, osteophyte formation and the development of subchondral sclerosis and cysts (Keyes *et al.*, 1992). Scoring systems for OA features in plain radiographs include those proposed by Kellgren and Lawrence (Kellgren & Lawrence, 1957), by Ahlbäck (Ahlbäck, 1968) and by the Osteoarthritis Research Society International (OARSI) (Altman & Gold, 2007). When compared to newer imaging modalities such as MRI, plain radiographs lack sensitivity and cannot detect localized cartilage damage (Kim *et al.*, 2003). Also the radiographic findings in plain radiographs may not correlate adequately with the severity of the symptoms (Creamer *et al.*, 2000). Nevertheless, plain radiographs are inexpensive and readily available, making them very useful in clinical and research settings.

Newer MRI techniques such as delayed gadolinium-enhanced MRI of the cartilage (dGEMRIC), T1rho, sodium MRI, T2 mapping and (Jazrawi *et al.*, 2011) are currently being used to study the early stages of OA. Physiological MRI methods such as dGEMRIC allow us to study the glycosaminoglycan (GAG) concentration of cartilage (Kim *et al.*, 2003) and have a good correlation with the histological grade of osteoarthritis (Zilkens *et al.*, 2013) and with the development of future knee OA (Owman *et al.*, 2008). The main downsides of this technique are the long scan times and the use of high doses of intravenous nephrotoxic contrast agent (Marckmann *et al.*, 2006). Advantages of T2 mapping are that it does not require contrast, has acceptable scanning times, and its values are correlated with histological changes (Nishioka *et al.*, 2012). Moreover, the addition of a T2 mapping sequence to a routine MRI improves the detection of knee cartilage lesions (Kijowski *et al.*, 2013).

There are several systems for grading histological changes in OA, the most widely

used being the Mankin (Mankin *et al.*, 1971) score system and the OARSI (Pritzker *et al.*, 2006). These systems focus on cellular changes, proteoglycan content and architectural changes of the articular cartilage (fissures, erosion, osteophyte formation). Since they rely on a tissue biopsy of the affected joint, and require a specialized and time-consuming tissue processing, their usefulness in the clinical setting is limited, being more often used in the research setting.

1.7 Treatment of osteoarthritis

The treatment of OA is diverse, ranging from patient education to surgical intervention, the main purpose being to prevent further cartilage damage and progression to end-stage disease that usually is accompanied by debilitating symptoms.

Lifestyle modifications

Many risk factors for OA are amenable to lifestyle changes (Messier *et al.*, 2004). For instance, weight loss in obese patients reduces the risk of developing OA and improves symptoms once the disease is established. Also, specific physical therapy, low-impact aerobic exercises and muscle strengthening improve symptoms in the early stages of OA (Richmond *et al.*, 2010). The use of a cane or a walker can also be beneficial by reducing the joint load and providing stability.

Pharmaceutical drugs

Medications currently used to treat patients are unable to reverse the cartilage damage and are used for treating the symptoms. Paracetamol and non-steroidal anti-inflammatory drugs (NSAID) are frequently used for symptom control as they provide good pain control (Richmond *et al.*, 2010; Hochberg *et al.*, 2012). However, there are some concerns over the long term use of NSAIDs due to the risk of gastrointestinal bleeding and of cardiovascular events. Glucosamine and chondroitin appear to have anti-inflammatory and anabolic properties in-vitro (Chan *et al.*, 2005), but the oral administration of these molecules does not yield clinically relevant effects (McAlindon *et al.*, 2000). Therefore, its use is not recommended in guidelines published by international bodies (Richmond *et al.*, 2010; Hochberg *et al.*, 2012; McAlindon *et al.*, 2014).

HA is a GAG found in the synovial fluid and acts as a lubricant. Intra-articular injection of this molecule has been used as viscosupplementation but no clinically relevant benefit has been found in terms of pain or function (Rutjes *et al.*, 2012). Intra-articular steroid injections are useful for short-term relief of pain and inflammation, but do not prevent cartilage degradation (Richmond *et al.*, 2010).

Surgery

Microfractures, cartilage transplantation and autologous chondrocyte transplantation are valid treatment options for small, localized, cartilage defects (Van Assche *et al.*, 2010).

However, the newly formed cartilage does not have the same mechanical properties as hyaline cartilage (Mollon *et al.*, 2013). Therefore, the relief provided by these treatments is temporary (Minas *et al.*, 2014).

An arthroscopic debridement of the joint is not recommended as a standard treatment in OA, except if there are associated intra-articular loose bodies or symptomatic meniscal lesions (Richmond *et al.*, 2010).

In selected cases, osteotomies are useful in restoring limb alignment and in unloading areas of damaged cartilage, effectively delaying the symptoms of late stage OA (Maistrelli *et al.*, 1990; Akizuki *et al.*, 2008).

Partial or total joint arthroplasties, replace the articular surfaces of a joint by metal, ceramic or polyethylene implants, are a reliable and cost-effective method of treating the symptoms of late stage OA (Chang *et al.*, 1996). While the longevity of the implants has improved, with 10-year survival rates reaching as high as 94.8% (94.4-95.2) for cemented hip implants, there is always the risk of reoperation for septic or aseptic failure of the implants (Malchau *et al.*, 2002).

1.8 Animal models in osteoarthritis research

Animal models are paramount research tools for studying the pathological processes and potential therapeutic targets for many diseases, including osteoarthritis as they allow control of a large number of variables in the disease process, and ideally offer a great degree of reproducibility.

The ideal animal model for the study of the human OA should be mammalian, inexpensive, easy to house and manage, with known genome and a wide array of available molecular tools. In addition, it should have a sufficient size as to make surgical interventions and collection of synovial fluid feasible; display a consistent and reproducible disease with a linear progression, occurring in a reasonable time frame; and recapitulate the human pathology in all tissues of the joint (Little & Smith, 2008). There are several animal models available but, at the moment, none fulfil all of the above requirements.

Mice (*Mus musculus*) models of OA have several advantages: they have a small size, are tractable animals and maintaining them in an animal facility is simpler and more economical when compared to larger animals such as rabbits, goats, sheep or horses. The ethical controversies involved are less than those when using cats, dogs or primates. Finally, their rate of breeding, maturation and disease onset is rapid.

Since the full genome of the mouse is known, it is possible to *(i)* design strains with targeted genetic modifications, *(ii)* design primers for the desired genes and *(iii)* perform microarrays or transcriptome studies.

One of their main advantages, is also one of the main drawbacks as their reduced size limits the discrimination and quantity of tissues, limits the volume of available synovial fluid and increases the difficulty of surgical procedures; also the joints have a much thinner articular cartilage compared to larger species making it more difficult to differentiate the different layers of the hyaline articular cartilage.

The methods of inducing OA are also varied and adequately selecting a method

is fundamental since it may or may not replicate the sequence of events leading to the disease in humans. For instance, injection of enzymes or other molecules in the joint may be a suitable model for inflammatory arthritis but a poor model for primary arthritis; different methods of induction can yield opposite results even when the same animal model is used (Little & Zaki, 2012).

In addition to the models where OA has a spontaneous onset secondary to the ageing process (Mason *et al.*, 2001; Stoop *et al.*, 1999), there are a variety of methods for inducing OA in mice (Pritzker, 1994; Little & Smith, 2008), such as genetic modification (Ameye & Young, 2002), intra-articular injection (van der Kraan *et al.*, 1990; van Beuningen *et al.*, 2000), or surgical intervention (Kamekura *et al.*, 2005; Glasson *et al.*, 2007).

Although there are different surgical methods for inducing OA in the mouse knee, the underlying concept is to destabilize the joint creating a mechanical overload that leads to cartilage destruction. To effectively study the pathological process of cartilage destruction using surgically induced models, a number of factors have to be considered; *(i)* a greater degree of instability causes a more severe and more rapidly progressing disease (Kamekura *et al.*, 2005), hence, if the objective is to study the initial phases of the disease process it is preferable to choose one of the milder methods, such as medial meniscectomy or meniscal destabilization (Glasson *et al.*, 2007) *(ii)* the strain of animal (Eltawil *et al.*, 2009) *(iii)* sex (Ma *et al.*, 2007) and *(iv)* age of the animal (Loeser *et al.*, 2012) at the time of surgery also influence the disease progression. In order to make meaningful comparisons between different experiments all of these variables have to be controlled.

Chapter 2

Aims of the study

The aims of the study can be described as follows:

1. Compare the morphological and histological features of surgically induced arthropathy between Hfe-KO and WT mice
2. Identify genes differentially expressed between WT and Hfe-KO mice knee joints following surgical induction of osteoarthritis
3. Evaluate the relationship between the different genotypes of the HFE gene and musculo-skeletal complications of hemochromatosis in humans

Chapter 3

Methods

3.1 Paper I

3.1.1 Animal model, experimental procedure, feeding and housing

This study protocol was approved by the Portuguese National Authority for Animal Health (DGAV ref. 0421/000/000/2013) and by the Ethics Committee of the University NOVA in Lisbon (nº 11/2013/CEFCM). Hfe-KO mice in a C57BL/6 background (Levy *et al.*, 1999) (Hfe-KO) were used as a model for human HH. A colony of these animals, as well as of wild-type (WT) C57Bl/6 mice used as control, is routinely maintained at the University of Algarve Animal Facility. Animals were kept in a 12hrs light/dark cycle, had access to water and food (SDS RM3A, iron content 161 mg/kg) ad-libitum from weaning up to time of euthanasia and maintained in specific pathogen-free conditions in individually ventilated cages with 4-6 animals each, according to the Animal Care and Use Committee protocols.

A total of 56 ten week-old male mice, 28 of each strain, were used and the allocation of animals, as summarised in Figure 3.1, was the following; twenty animals for histological grading of cartilage destruction; ten animals for micro-CT and assessment of iron deposits in the knee joint; twenty animals for assessment of gene expression, of which twelve were also used for determining iron liver concentration and serum iron parameters; and six animals were used for immuno-histochemical staining.

Osteoarthritis was induced by medial meniscectomy and medial collateral ligament transection, as previously described by Kamekura (Kamekura *et al.*, 2005). Briefly, ten week-old male mice were anaesthetised with a combination of ketamine (1mg/10g body weight , i.p., Merial France), xylazine (0.1mg/10g body weight, i.p., Bayer, Germany) and acepromazine (0.03mg/10g body weight, i.p., Vétoquinol, France)(Buitrago *et al.*, 2008). Using a stereomicroscope (Nikon SMZ1500) and microsurgical instruments a medial knee arthrotomy was performed and the patella was externally subluxated, the right knee medial collateral ligament was transected and the medial meniscus removed (MNX) (Figure 3.2). The left knee was sham-operated (SHAM), i.e., the arthrotomy and external subluxation of the patella were performed but no ligament transection or

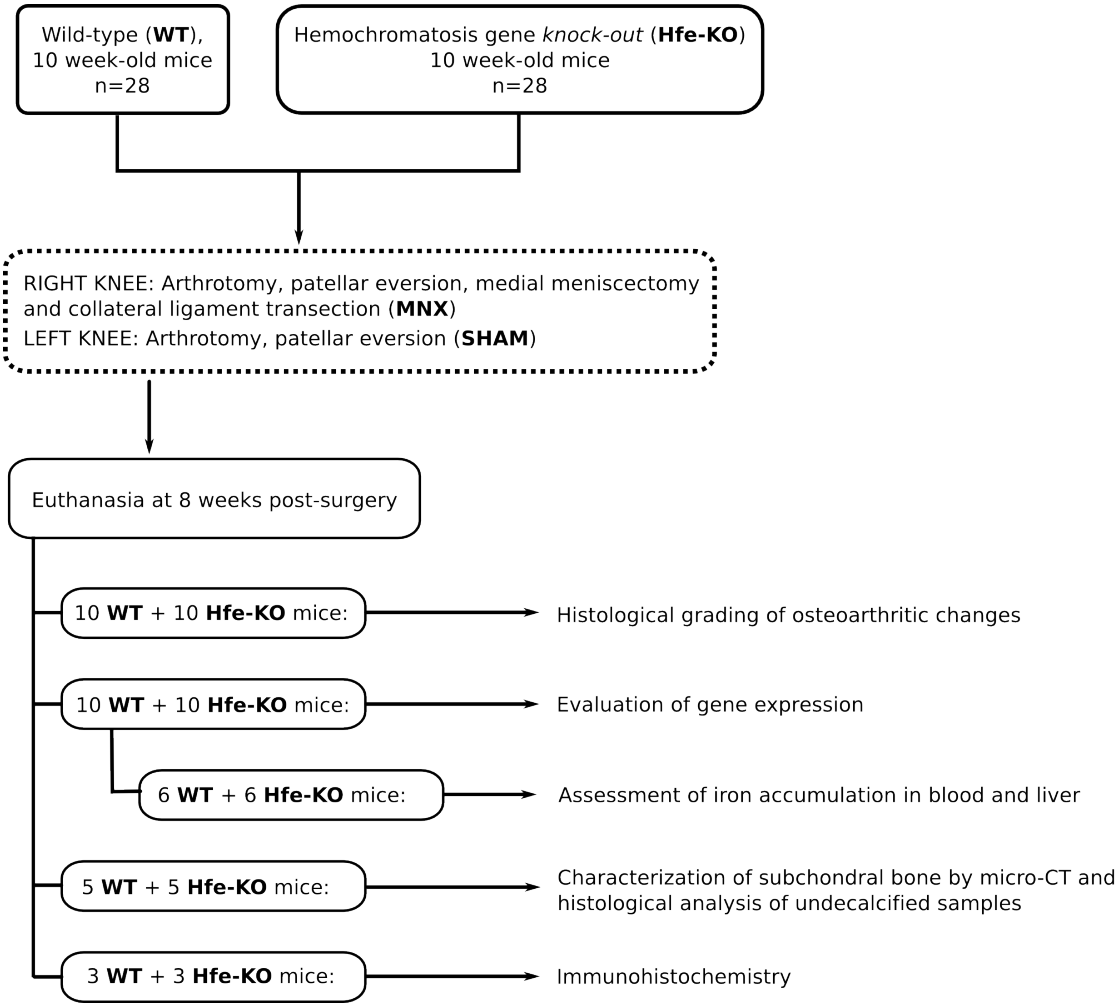

Figure 3.1: Flowchart describing the allocation of animals in the different experiments

meniscal excision were made. Care was taken to prevent damage or desiccation of the articular cartilage. The joint was then irrigated with sterile saline, the articular capsule closed with 8-0 absorbable suture (Vicryl, Ethicon, USA) and the skin was closed with 6-0 non-absorbable sutures (Ethilon, Ethicon, USA).

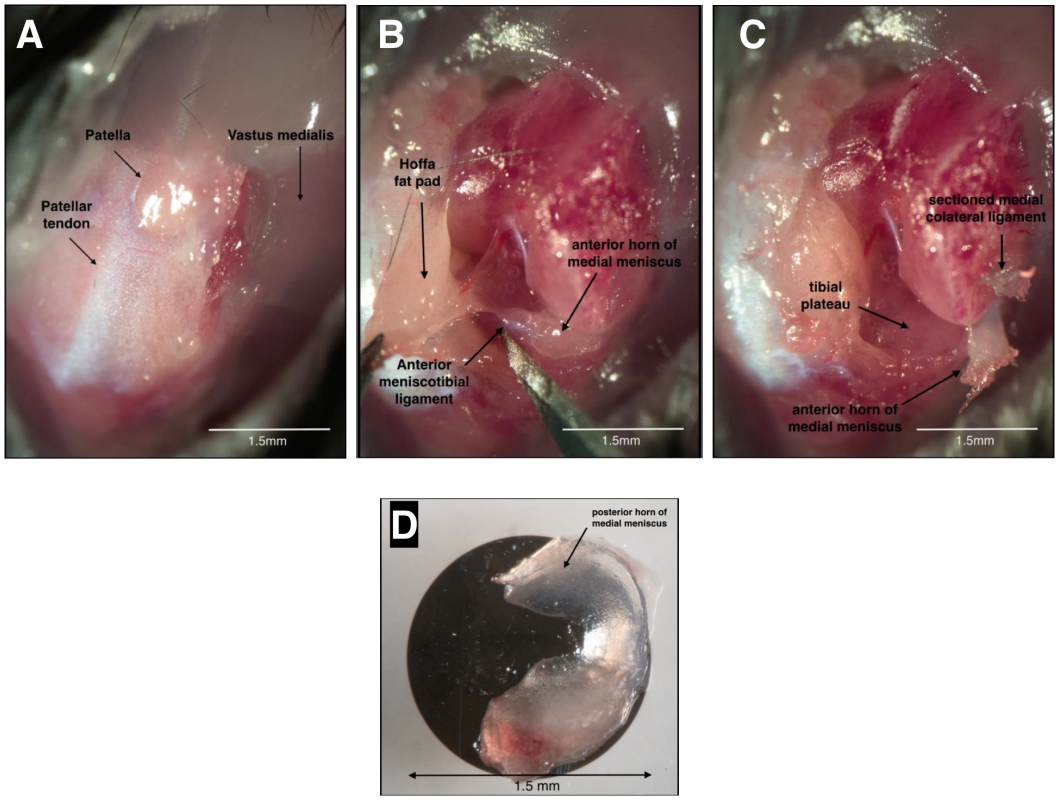

Figure 3.2: Surgical induction of osteoarthritis in the mouse knee. A) Knee joint after exposure of subcutaneous tissue and medial arthrotomy. B) Knee joint following lateral subluxation of the patella, exposing the anterior horn of the medial meniscus. C) Excision of the medial meniscus after sectioning the medial collateral ligament and the anterior tibio-meniscal ligament. D) Medial meniscus after excision. Images taken through the stereomicroscope with Olympus OM-D E-M5 camera, Carl Zeiss universal digital camera adapter d30 and Olympus 60mm MACRO.

All surgical procedures were performed by the author, who is credited by the Portuguese National Authority for Animal Health as competent to perform experiments with laboratory animals (DGAV ref. 0421/000/000/2013). The procedures were carried out during the morning and the surgeon was blinded to the strain of the animals. Animals were euthanized 8 weeks after surgery and bilateral knee joints were collected and processed as described in the next sections. There were no surgery-induced mortality nor adverse effects and all of the operated animals were included in the analysis.

3.1.2 Assessment of iron accumulation

Unless noted otherwise all the reagents used were purchased from Sigma-Aldrich. For the assessment of iron accumulation in the liver and blood, samples of 6 animals of each strain were used. Hepatic non-heme iron concentration in µg of iron per mg of wet liver weight was determined as previously described (Rebouche *et al.*, 2004). Briefly, a sample of liver was collected, homogenized in a 1:10 dilution (weight/volume), then equal volumes of the tissue homogenates and protein precipitation solution ($1\,\mathrm{mol\,L^{-1}}$ HCl and 10% trichloroacetic acid) were mixed in "boil-proof" tubes and heated to 95ºC for 1h. After centrifuging for 10min the supernatant was collected and an aliquot was mixed with an equal volume of chromogen solution ($0.508\,\mathrm{mmol\,L^{-1}}$ of ferrozine, $1.5\,\mathrm{mol\,L^{-1}}$ sodium acetate and 10% L-ascorbic acid). After 30min at room temperature the absorbance at 562 nm was measured and the concentration calculated using a calibration curve prepared on the same day from $FeCl_3$ stock solution [$1\,\mathrm{mg\,mL^{-1}}$] and diluted to 5, 10, 15, 25 and 50 $\mu\mathrm{g\,mL^{-1}}$ of iron. At the time of euthanasia blood was collected by intracardiac puncture, in tubes containing Heparin-Lithium, and sent to an external certified commercial laboratory (DNAtech, Lisbon, Portugal) for determination of serum iron, serum ferritin and also serum transferrin saturation.

3.1.3 Grading of osteoarthritic changes

Bilateral knee joints from 10 animals in each group were isolated, cleaned of adherent soft tissues in ice-cold phosphate buffer saline (PBS) and fixed for 24hrs in 4% paraformaldehyde in PBS pH7.4, followed by decalcification with $0.5\,\mathrm{mol\,L^{-1}}$ ethylenedinitrilotetraacetic acid (EDTA) in PBS pH 7.4 for 3 weeks. After dehydration through graded alcohols and inclusion in paraffin, 5µm sagittal sections were cut from the medial compartment of the joints, three sections per level with a 150µm interval between levels, on a rotary microtome (Microm HM340E, Germany) and stained with Safranin-O/Fast Green/Meyer's Hematoxylin. Two separate observers, without knowledge of the strain and intervention, graded the cartilage lesions using the semi-quantitative scoring system proposed by Glasson et al. (Glasson *et al.*, 2010), also known as the OARSI scoring system for murine osteoarthritis. This system is based on the cartilage destruction extent, graded 0-6 depending on the depth of the lesion and on the percentage of the articular surface affected. The medial tibial plateau and the medial femoral condyle were graded separately. The score for each knee was the sum of the scores from each of the three sections. The final score for each joint was obtained by averaging both observers' score

for that joint.

3.1.4 Morphological characterization of the knee joint by micro-CT

Micro-CT was performed on bilateral knee joints of 5 animals in each group with a Skyscan 1172 X-ray computed microtomograph (Bruker, Belgium) prior to inclusion in methyl methacrylate (MMA). The samples were wrapped in laboratory film (Parafilm) and placed inside an empty 1.5ml tube to prevent desiccation. The tubes were fixed on the sample holder with adhesive plasticine. For the image acquisition the following parameters were used: X-ray tube potential 70 kVp, X-ray tube current 100µA, 0.5mm Al filter, rotation step 0.4^{o}, isotropic voxel size $5\mu m^3$, integration time 500ms, frame averaging = 6. For reconstruction we used NRecon software (v1.6.9.8, Bruker, Belgium) with Gaussian smoothing, ring artefact correction and 40% beam hardening correction applied. Using Dataviewer software (v1.4.4, Bruker, Belgium) orthogonal sagittal projections of the entire internal compartment of the knee, from the medial border of the internal tibial plateau to the tibial insertion of the posterior cruciate ligament, were generated and then exported to CTAn software (v1.13.11.0, Bruker, Belgium) for further analysis. Coronal sections of the whole joint were also generated to assess the presence of osteophytes. The proximal epiphysis of the tibia was selected as a region of interest (ROI). To quantify the characteristics of the subchondral bone (Botter *et al.*, 2006) the epiphysis was further divided into a cortical part (subchondral bone plate) and a trabecular part, by manually drawing an irregular anatomic contour adjacent to the endocortical surface at intervals of 15 sections, and the software calculated the intermediary contours by interpolation. These parts were analysed separately (Figure 3.3). The greyscale images were segmented, using the same manually chosen global threshold for all the samples. The following three dimensional (3D) morphometric parameters were provided by the software and used to describe the bone of the trabecular compartment: Bone volume fraction (BV/TV, in %) is the ratio of the segmented bone volume to the total volume of the region of interest; Connective density (Conn.D, in mm^{-3}) is a measure of the average number of trabeculae per unit of volume; Trabecular thickness (Tb.Th, in µm) is the mean thickness of trabeculae;. To describe the subchondral bone plate we measured the average thickness (Sb.plate.Th, in µm).

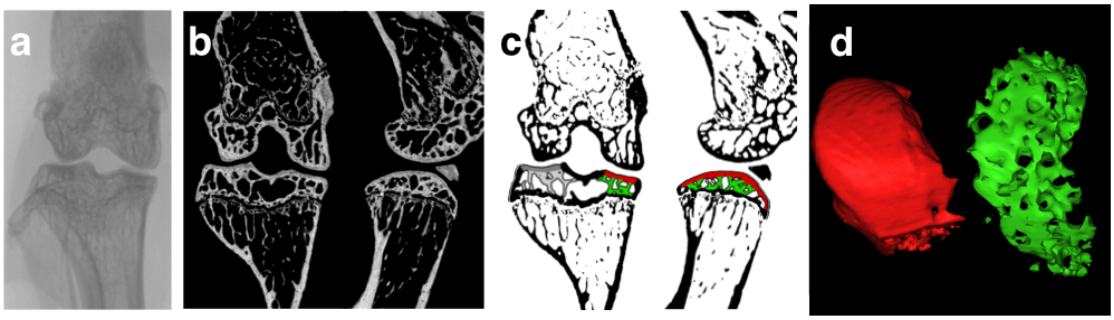

Figure 3.3: Process of micro-CT acquisition and analysis. The digitized image a) is reconstructed and resliced in orthogonal sagittal and coronal projections b), using a predefined threshold, the grayscale images are binarized and the ROIs are selected c), in this case the ROIs are the medial tibial subchondral plate (red) and the medial tibial subchondral trabecula (green), afterwards d) the software generates a 3D model and calculates the desired parameters.

3.1.5 Histological evaluation of undecalcified samples

Following micro-CT the samples were included in MMA at 4ºC. From these undecalcified samples, 5µm sagittal sections were cut from the medial knee compartment, eight sections per level with a 50µm interval between levels, on a heavy-duty microtome (Leica SM2500S, Germany) equipped with 40º wedge angle, profile-D, tungsten carbide knives. Sections were stained with Perl's and counterstained with Neutral Red in order to assess iron deposition. The ROIs for this analysis were the knee joint and the proximal epiphysis of the tibia. The total area of hemosiderin deposits in the synovial membrane was measured as previously described (Heiland *et al.*, 2010). Measurements were made using a semi-automatic image analysis software (ImageJ v1.49). Sections were also stained with aniline blue / Ponceau fuchsin in order to evaluate the articular calcified cartilage. After capturing high-magnification images of the samples we used image analysis software (ImageJ v1.49) to create a high-resolution panorama of the medial tibial plateu of each section stained with aniline blue/Ponceau fuchsin, and measure the area stained in pink that corresponds to the hyaline articular cartilage (HAC) and the area stained in light blue that corresponds to the articular calcified cartilage (ACC). We then determined the ratio of HAC/ACC area and the average height of the ACC (Figure 3.4.

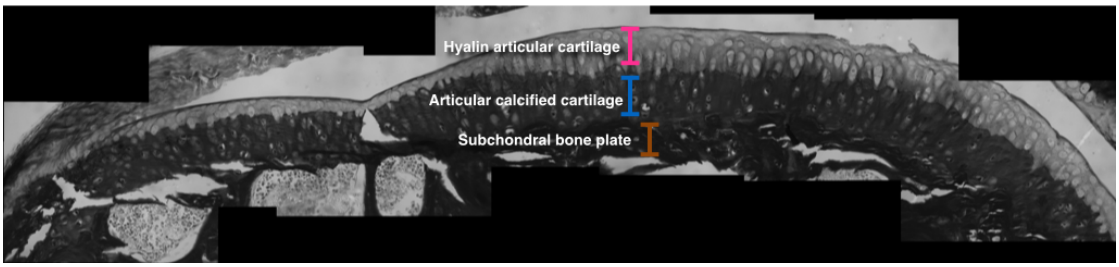

Figure 3.4: Measurement of calcified cartilage. Several high magnification microphotographs were stitched with ImageJ plugin TrakEM2, resulting in a high resolution mosaic encompassing the entire articular surface of the section of the medial tibial compartment. The image was then converted to grayscale where the differences between ACC, HAC and subchondral bone are visible and, using ImageJ, the area and height of each layer was measured.

3.1.6 Evaluation of gene expression

The bilateral knee joints of ten mice from each strain were used for ribonucleic acid (RNA) isolation. Immediately after euthanasia, both knee joints were immersed in RNALater at 4^{o}C and cleaned of soft tissues including ligaments, menisci and synovial membrane, in order to isolate the tibial and femoral cartilage and subchondral bone of the medial knee compartment (Figure 3.5).

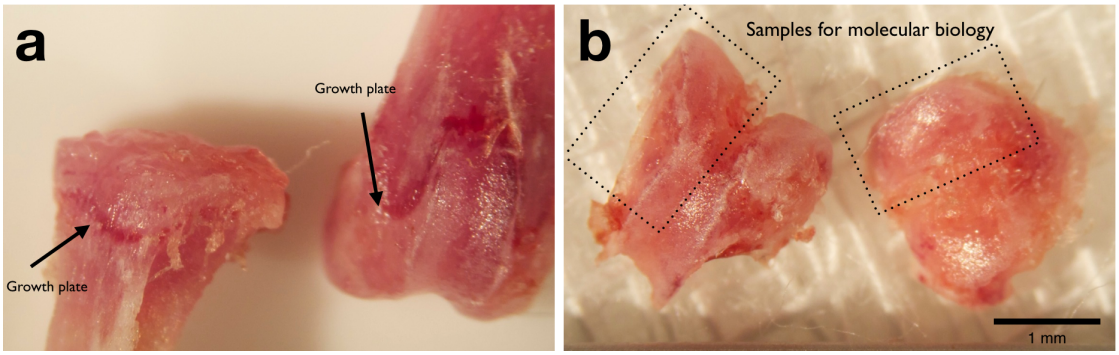

Figure 3.5: Samples for molecular biology. a) After euthanasia the joints were cleaned of soft tissues and the epiphysis was separated from the metaphysis at the level of the growth plate (arrow); b) the epiphysis was further separated in medial and lateral compartments (dotted box). The medial compartments were used for evaluation of gene expression and the lateral compartment was discarded. Images taken through the stereomicroscope with Olympus OM-D E-M5 camera, Carl Zeiss universal digital camera adapter d30 and Olympus 60mm MACRO.

The samples were then separated into four groups, WT.SHAM, Hfe-KO.SHAM, WT.MNX, Hfe-KO.MNX according to the strain of the animal (WT or Hfe-KO) and the knee (MNX or SHAM) from which the sample originated. In order to obtain enough

messenger RNA (mRNA) to perform the analysis, the samples were pooled, each pool containing samples from two knees of the same group, adding to a total of 5 pools in each group. The pooled samples were stored in RNALater at 4ºC for 24hrs. Afterwards the excess RNALater was removed and samples were stored at -80ºC until further processing. The tissue pools were homogenized using a Precellys 24 tissue homogenizer and reinforced tubes with 2.8mm steel beads. RNA was extracted using TRI Reagent according to the manufacturer's indications. RNA was purified using the High Pure RNA Isolation Kit (Roche) and quantified with an Experion RNA analysis system (Bio-Rad). Only samples with RNA quality indicator (RQI) >7 were used for the reverse transcription (RT) and subsequent analysis. The RT reaction was carried out using 0.5µg of total RNA per reaction with the Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Invitrogen) according to the manufacturer's indications. Two negative control reactions were prepared, one without template and the other without the M-MLV reverse transcriptase. Using the Primer Premier v5.0 software, primers were designed for the following mouse genes;

Aggrecan (Acan)
A disintegrin-like and metallopeptidase with thrombospondin type 1 motif, 5 (Adamts5)
Bone morphogenetic protein 6 (Bmp6)
Collagen, type X, alpha 1 (Col10a1)
Collagen, type II, alpha 1 (Col2a1)
Hemochromatosis (Hfe)
Interleukin 1 beta (Il1b)
Interleukin 6 (Il6)
Matrix metallopeptidase 3 (Mmp3)
Matrix metallopeptidase 13 (Mmp13)
Runt related transcription factor 2 (Runx2)
Transferrin receptor (Tfrc)

The corresponding sequences are in Table 3.1. The real-time polymerase chain reaction (RT-PCR) efficiency for each gene was found to have <10% deviation between each other (Schmittgen & Livak, 2008). Gene relative expression was determined using the 2 -ΔΔCq method (Livak & Schmittgen, 2001). Each RT-PCR was carried out using 50ng of complementary deoxyribonucleic acid (cDNA), 10µl SsoFast™ EvaGreen® Supermix (Bio-Rad), 300mmol l^{-1} of primers in a volume completed to 20µl with high-purity water free of DNase and RNase. Amplifications were performed in a Bio-Rad CFX-96 machine. The RT-PCR was carried out for 40 cycles using the parameters specified by the manufacturer for the combination of machine and master mix. Each reaction was carried out in triplicate and the final Cq value of each biological replicate was the average of the technical triplicates. The ribosomal protein L13A (Rpl13a) gene was used as a control of endogenous gene expression (Curtis *et al.*, 2010) and the WT.SHAM group as the reference condition.

Table 3.1: Sequence and reference of primers used in the RT-PCR amplification reactions

Gene	NCBI RefSeq	Forward primer (5'->3')	Reverse primer (5'->3')
Adamts5	NM_011782.2	TCAGCCACCATCACAGAA	CCAGGGCACACCGAGTA
Acan	NM_007424.2	CAGGGTTCCCAGTGTTCAGT	CTGCTCCCAGTCTAACTCC
Bmp6	NM_007556.2	TTGAACCGCAAGAGTCTCCTGG	TGTGGGGAGAACTCCTTGTCGT
Col2a1	NM_001113515.2	CCAACACCGCTAACG	GGTCTTGCCCCACTTAC
Col10a1	NM_009925.4	AAGGAGTGCCTGGACACAAT	GTCGTAATGCTGCTGCCTAT
Hfe	NM_010424.4	CTTCAGTCGGTCTCCGTAAAAC	GTGACTCCACTGATGATTCCG
Il1b	NM_008361.3	AAAGTATGGGCTGGACTGTTTCTAA	TTCTTGTGACCCTGAGCGACC
Il6	NM_031168.1	GTTCTCTGGGAAATCGTGGA	CTCTGGCTTTGTCTTTCTTGT
Mmp3	NM_010809.1	ATGAAAATGAAGGGTCTTCCGG	GCAGAAGCTCCATACCAGCA
Mmp13	NM_008607.2	TGATGGCACTGCTGACATCAT	TGTAGCCTTTGGAACTGCTT
Rpl13a	NM_009438.5	GGATCCCTCCACCCTATGACA	AGCCGAACAACCTTGAGAGC
Runx2	NM_001146038.2	GAGCGGACGAGGCAAGAGTTTCACC	GAGGCGGGACACCTACTCTCATACTGG
Tfrc	NM_011638.4	GCAGCATTGGTCAAAACATGG	GCTTTGGGCATTTGCAACCT

3.1.7 Immunohistochemistry

Bilateral knee joints of three mice from each strain were used for immunohistochemical localization of a disintegrin and metallopeptidase with thrombospondin motifs 5 (ADAMTS5), type X collagen, MMP3 and matrix metallopeptidase 13 (MMP13). The samples were fixed, decalcified and embedded in paraffin as described above. After dewaxing and rehydration, epitope retrieval was performed by heating the sections at 80^{o}C for 25minutes in Tris-EDTA pH 9 buffer. To increase the permeability of the extracellular matrix, sections were incubated with 2.5% hyaluronidase for 30 min at 37^{o}C as previously described (Kamekura *et al.*, 2005). After blocking for 30min at room temperature with Tris buffered saline (TBS) containing 5% goat serum + 1% bovine serum albumin, the sections were incubated with polyclonal rabbit antibodies against mouse MMP3, MMP13 (Proteintech, England) , ADAMTS5 (Abcam ab41037) at a dilution of 1:40 and against type X collagen (Abcam ab58632) at a dilution of 1:20 for 24 h at 4^{o}C. The sections were then treated with 0.3% hydrogen peroxide in TBS for 15 min to block endogenous peroxidases, rinsed with TBS + 0.05% Tween-20 and incubated with horseradish peroxidase-conjugated goat antibodies against rabbit IgG (Sigma-Aldrich) at a 1:100 dilution for 1 h at room temperature. After rinsing with TBS the sections were covered with a 0.05% 3,3'-diaminobenzidine solution for 5 min at room temperature to visualize the location of the target protein-primary-secondary antibodies complex, counterstained for 30s with Mayer's haematoxylin, left to air-dry and then mounted in DPX mounting medium.

3.1.8 Statistical analysis

Unless stated otherwise, results are expressed as the mean and corresponding 95% Confidence Interval (CI). The bar and range plots were generated to observe the distribution of the mean (bar) and corresponding 95% CI (upper and lower range). In the instances where the sample size in each group is smaller than n=10 scatter plots were generated instead of the bar plots. The Student's-t test was used to compare continuous variables between different strains. The non-parametric Mann-Whitney U test was used to compare differences between ordinal variables or when the assumptions of normality and homoscedasticity were not met. Pairwise comparisons using paired t-tests with Holm's P-value adjustment were used to compare continuous variables between different groups (WT.SHAM vs. WT.MNX vs. Hfe-KO.MNX vs. Hfe-KO.SHAM), in order to account for the correlation between joints of the same animal. The Wilcoxon matched pairs test with Holm's P-value adjustment was used for ordinal variables or when the assumptions of parametric tests were not met. A two-tailed P-value <0.05 was considered statistically significant. All analysis and plotting were conducted using the R v3.1.1 (R Foundation for Statistical Computing, Vienna, Austria) software with *base, stats, plyr, reshape2 and ggplots2* packages.

3.2 Paper II

3.2.1 Patient recruitment and evaluation

Patients who were members of the Association Hémochromatose France (AHF) completed a self-administered questionnaire that has previously been described elsewhere (Richette *et al.*, 2010). Patients were informed of the purpose of the study and gave their informed consent. This study was conducted in accordance with the recommendations of the Helsinki Declaration. The Institutional Review Board (n°IRB00006477-Comitéd'évaluation de l'éthique des projets de recherché biomédicale du CHU Nord-Hôpital Bichat, Paris) has reviewed and approved this study (n°10-074). We collected the following data: demographic characteristics (age, sex, weight, height, smoking habit, menopausal status); details of HH history (HFE genotype, ferritin level and transferrin saturation at diagnosis, symptoms before diagnosis); general clinical features (asthenia, diabetes mellitus, cardiac disease); joint and spine involvement (patients were asked if they had received a diagnosis of OA by a physician; if they had ever complained of low back pain or sciatica; if they had knee-, hip-, or ankle-replacement prosthesis); bone involvement (patients were asked if they had received a diagnosis of osteoporosis (OP) by a physician and if they had a history of fractures). To confirm the validity of the genotype reported by patients, we reviewed the medical records of a sample of 20 patients followed in P. Richette's department and who had reported having homozygosity (n=10) and duplex heterozygosity (n=10). The rate of concordance was 95% (19 of 20). One patient who declared homozygosity was actually duplex heterozygous.

3.2.2 Statistical analysis

Continuous variables were tested for normality and homoscedasticity. All continuous variables in our sample were non-normally distributed (skewed) and were described using the median and interquartile range (IQR). Differences between groups were assessed using the Mann-Whitney test. Association between categorical variables was tested using the chi-squared test. Crude odds ratios (OR) were calculated using a univariate logistic regression model, with the patient genotype (C282Y/C282Y vs. C282Y/H63D) as the independent variable and the outcome of interest as the dependent variable. Adjusted odds ratios (aOR) were calculated using a multiple logistic regression model, with the patient genotype and clinically relevant confounders (Richette *et al.*, 2010) as independent variables and the outcome of interest as the dependent variable. The design of our study precluded the assessment of iron overload by liver biopsy; therefore, we used ferritin level as a surrogate marker, with severity of iron overload defined by serum ferritin level >1000 µg/L at diagnosis. This cut-off has previously been found to be clinically appropriate (Allen *et al.*, 2010). All statistical analyses were carried out using Stata 13.1 (StataCorp, College Station, TX). A 2-tailed $p < 0.05$ was considered statistically significant.

Chapter 4

Results

4.1 Paper I

All the animals initially operated were included in the final analysis; the experimental procedure had no adverse effects on the mice.

Mice not expressing the Hfe allele have increased iron accumulation in the serum, liver and knee synovial membrane

The Hfe-KO mice had a significant plasmatic iron overload (n=6 per group, Figure 4.3A) and up to a 5 fold increase in hepatic iron content when compared to their controls [1121, 95% CI (927, 1316) µg iron/mg of wet liver weight vs. 282, 95% CI (196, 367) µg iron/mg of wet liver weight; P-value =0.002, respectively, n=6 per group].

To evaluate the iron accumulation in the joints of Hfe-KO mice we used undecalcified, MMA-embedded sections of the knee, stained with Perl's. We observed hemosiderin deposits in the synovial membrane of the knee but not in the cartilage (Figure 4.1. These occupied a significantly greater area in the Hfe-KO mice, although there was no significant difference between the MNX and the SHAM operated sides (n=5 per group, Figure 4.3B). Unpublished data from our research team (Simão M) showed that in non-operated knees of Hfe-KO mice there is no accumulation of hemosiderin in the synovial membrane at 18 weeks of age (Figure 4.2).

These observations suggest that the hemosiderin deposits originate from the blood that enters the joint following the arthrotomy. The higher iron content of the Hfe-KO mice blood could explain the greater hemosiderin accumulation.

Using RNA extracted from the subchondral bone and articular cartilage we examined the Hfe and Tfrc mRNA expression. RT-PCR analysis confirmed the decreased Hfe expression in the Hfe-KO mice (n=5 per group, Figure 4.3C). The Tfrc expression was significantly decreased in the Hfe-KO SHAM-operated knees and was elevated in both strains on the MNX-operated knees. (n=5 per group, Figure 4.3D).

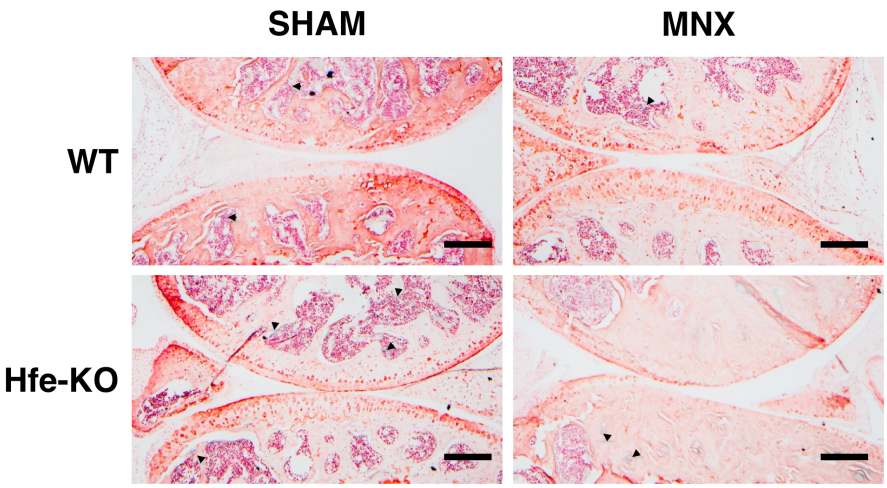

Figure 4.1: Iron accumulation in the knee joints of WT and Hfe-KO mice. Section of undecalcified , MMA embedded, knee medial compartment, stained by Perl's method and counterstained with Neutral Red. Note the absence of hemosiderin deposits (stained in blue) in the cartilage, but visible on the medullary space (arrowheads). Scale bar 200 µm.

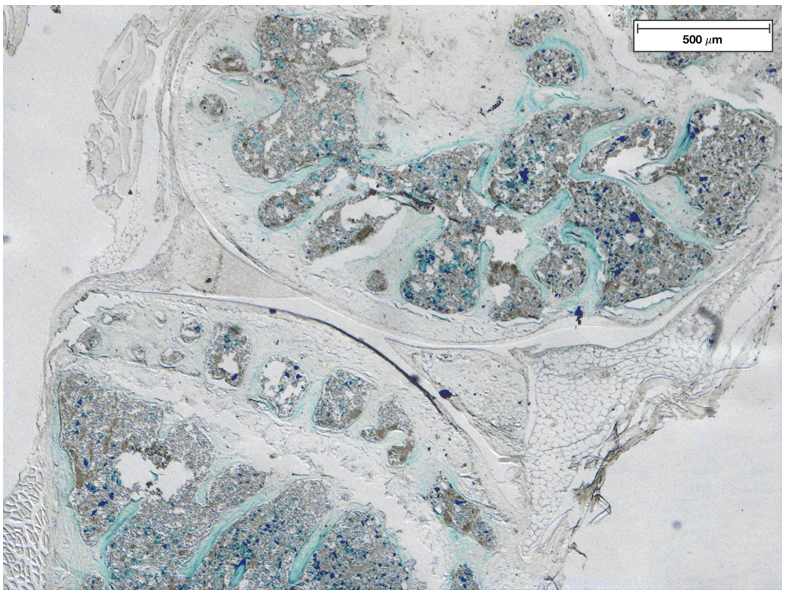

Figure 4.2: Iron accumulation in the knee joint of non-operated Hfe-KO mice. Section of undecalcified , MMA embedded, knee medial compartment, stained by Perl's method. Note the absence of hemosiderin deposits (stained in blue) in the cartilage and synovial tissue, but obvious in bone and medullary space.

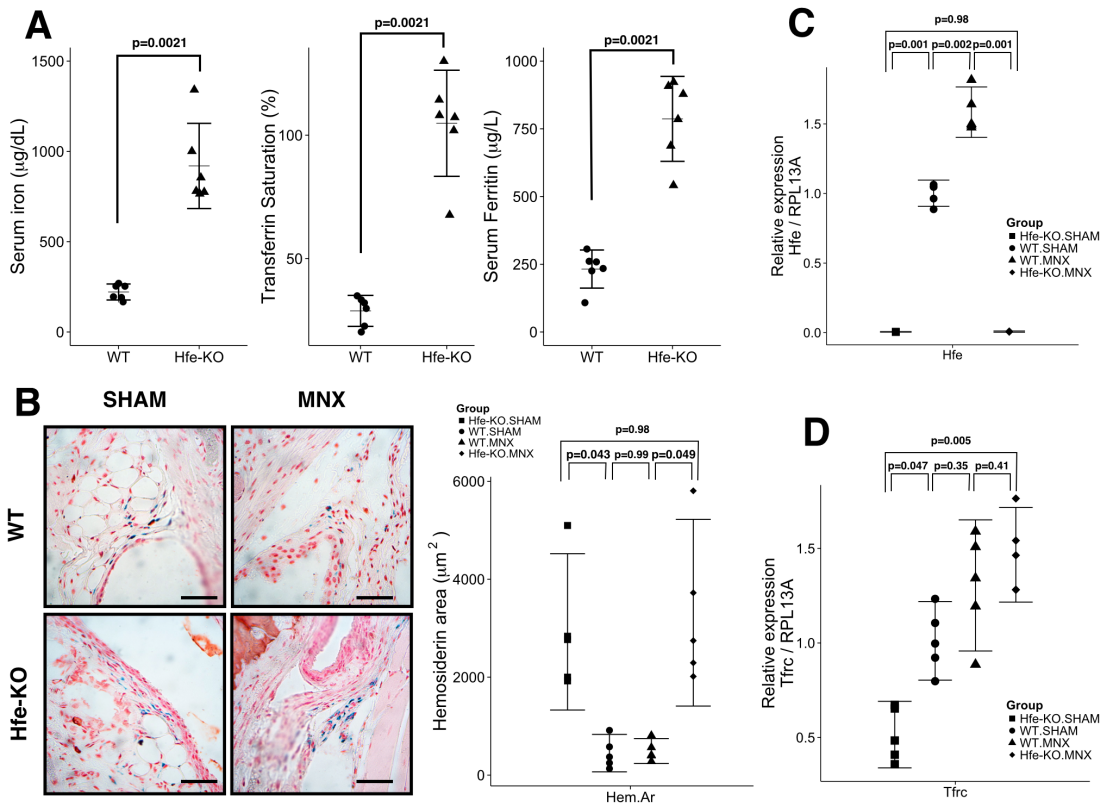

Figure 4.3: Iron parameters in WT and Hfe-KO mice (A) Bar plots for the different serum iron parameters. (B) Perl´s staining of MMA-embedded knee joint, high magnification view of the knee synovial membrane to observe the hemosiderin deposits (blue) and the quantification of their area showing a greater accumulation of iron in the synovial membrane of the Hfe-KO mice. Scale bar 50 µm. (C) Real time PCR, using mRNA isolated from the medial knee compartment of 18 weeks-old animals, to detect the expression of the Hfe gene in the Hfe-KO and control animals (n=5 per group). The results confirm that the Hfe gene is not expressed in the bone and cartilage of the Hfe-KO mice. (D) RT-PCR, using mRNA isolated from the medial knee compartment of 18 weeks-old animals, to detect the expression of the Tfrc gene. The results show that in Hfe-KO there is an underexpression of the Tfrc gene and that the medial meniscectomy leads to an overexpression of this gene. Data are shown as individual values (shapes) with the corresponding 95% CI (range).

Hfe-KO mice have increased cartilage degeneration and subchondral bone volume following the surgical induction of OA

The Hfe-KO mice were morphologically indistinguishable from their WT counterparts and had similar body weight at the time of euthanasia [24.5, 95% CI (23.4, 25.6)g vs. 25.2, 95% CI (24.3, 26.1) g respectively; P-value=0.33; n=28 per strain]. To evaluate the effect of the iron overload in the development of OA we surgically induced OA in Hfe-KO mice and in WT controls, using a previously described technique (Figure 3.2) (Kamekura *et al.*, 2005). At 8 weeks after the intervention the MNX-operated knees of the Hfe-KO mice showed a higher level of cartilage destruction (Figure 4.4A), resulting in significantly higher summed tibial and femoral OOARSI scores for the medial femoral condyle and medial tibial plateau (n=10 per group, Figure 4.4B).

We assessed the ACC and the HAC of the tibial plateau in the undecalcified, MMA-embedded knee sections. The MNX operated knees had a significantly lower ACC thickness, lower HAC/ACC ratio and greater subchondral bone height (n=5 per group, Figure 4.5).

To better evaluate the subchondral bone architecture of the medial tibial plateau, we performed micro-CT scans of the knee joints (Figure 4.6A). The MNX-operated knees in both strains showed an increase in bone volume, in trabecular thickness and in subchondral plate thickness, these parameters were significantly greater in the Hfe-KO mice (n=5 per group, Figure 4.6B).

Using RNA extracted from the subchondral bone and articular cartilage, we examined Bmp6 expression, a signalling molecule involved in iron homeostasis (Andriopoulos *et al.*, 2009) and bone formation (Kugimiya *et al.*, 2005) and found it to be increased in the Hfe-KO MNX operated knees compared to their WT controls (n=5 per group, figure 4.6C). These findings suggest that the Hfe-KO animals have an accelerated progression of the surgically induced OA, when compared to their WT controls.

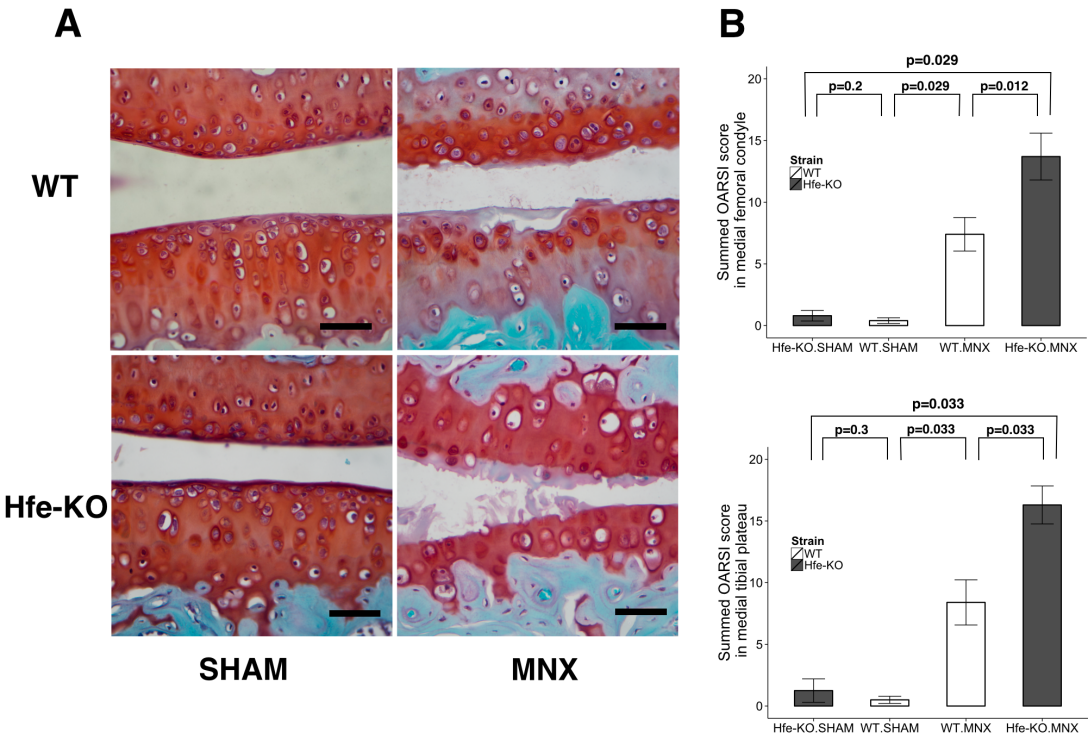

Figure 4.4: Cartilage changes following meniscectomy in the knee joints of WT and Hfe-KO mice (A) Haematoxylin-Fast Green-Safranin-O staining of the medial femoral condyle and tibial plateu of control (n=10 per group) and Hfe-KO mice (n=10 per group) at 8 weeks after surgical induction of OA. Scale bar 50 µm. (B) The Osteoarthritis Research Society International (OARSI) scores for the medial femoral condyle and medial tibial plateu of control and Hfe-KO mice (SHAM and MNX operated knees) were obtained by summing the score of three sections from the lateral, middle and medial one-third of the medial knee compartment from each mouse. Data are shown as the mean (bar) and corresponding 95% CI (range).

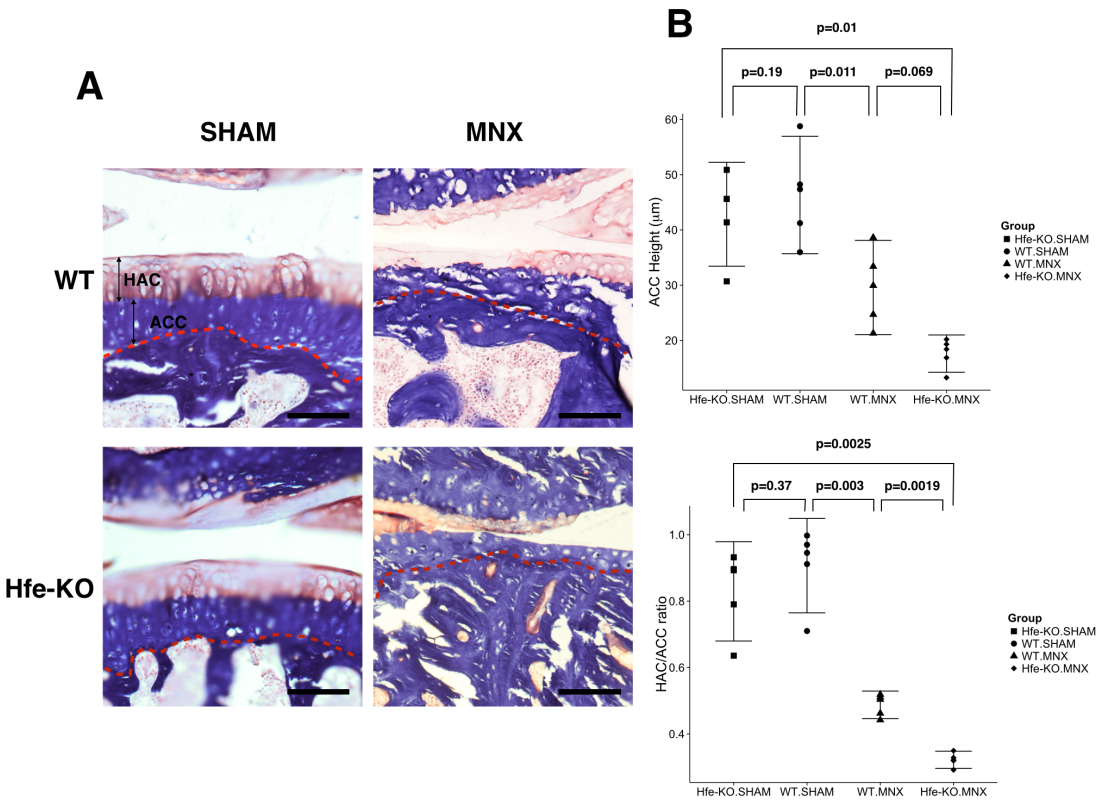

Figure 4.5: Articular cartilage calcification in the knee joints of WT and Hfe-KO mice. (A) Ponceau Fuchsin- Aniline Blue staining of undecalcified, MMA embedded, sections of the medial knee compartment of control and Hfe-KO mice (n=5 per group) at 8 weeks after induction of experimental OA. The arrows show the height of the pink coloured hyaline articular cartilage (HAC) and of the blue articular calcified cartilage (ACC). The dotted red line is the limit between the ACC and the subchondral bone. The MNX knees show a reduction of both ACC and HAC and increased subchondral bone thickness. Scale bar 100 µm. (B) Bar plots representing the mean height of the ACC and the ratio between HAC area /ACC area in the different groups (n=5 per group). The MNX surgery leads to a loss of both ACC and HAC, more pronounced on the Hfe-KO mice. Data are shown as the individual values (shapes) and corresponding 95% CI (range).

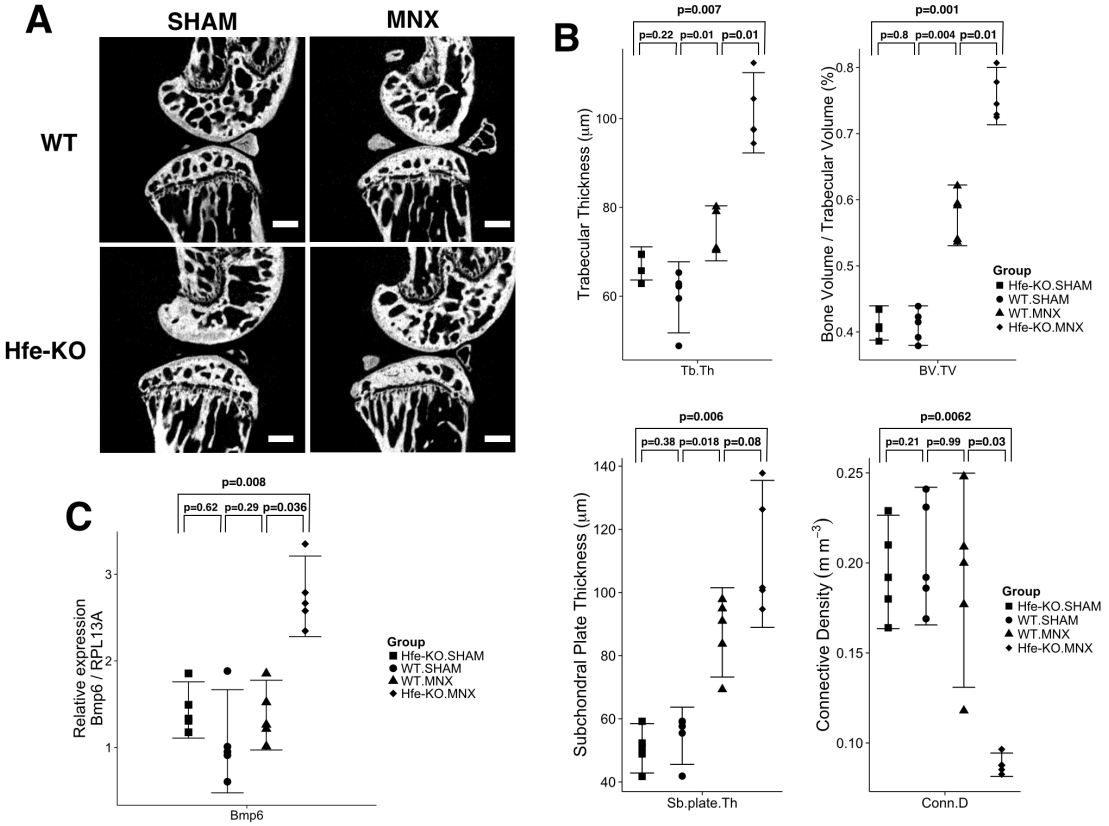

Figure 4.6: Subchondral bone changes following meniscectomy in WT and Hfe-KO mice (A) Knee micro-CT sagittal reconstructions at the level of the middle one-third medial tibial plateu of control (n=5 per group) and Hfe-KO mice (n=5 per group) at 8 weeks after surgical induction of OA. Scale bar 500 µm (B) Quantification of microarchitecture parameters of the medial tibial plateu of mice knees (n=5 per group). The results show that the MNX operated knees of the Hfe-KO animals have a greater degree of subchondral sclerosis when compared to their WT controls. Data are shown as the individual values (shapes) with the corresponding 95% CI (range) (C) RT-PCR, using mRNA isolated from the medial knee compartment of 18 weeks-old animals, to detect the expression of the Bmp6 gene. The results show that MNX surgery leads to an increase in the expression of the gene but only in Hfe-KO mice is it significant. Data are shown as the individual values (shapes) with the corresponding 95% CI (range).

The expression of genes related to extracellular matrix synthesis, chondrocyte hypertrophy and cartilage degradation are increased in knee joints of the Hfe-KO mice

To have a better understanding of the accelerated progression of the articular damage in the Hfe-KO mice, we investigated the expression of genes known to be involved in the acute inflammatory response and in the development of OA, namely genes coding for components of the extracellular matrix, chondrocyte hypertrophic markers, factors that promote bone formation, cartilage-degrading enzymes and cytokines. Although cytokines Il1b and Il6 showed no significant change in expression at 8-weeks post surgery (Figure 4.7), the remaining genes were up regulated in all MNX-operated knees (n=5 per group, Figure 4.8). Only Mmp3, a cartilage-degrading enzyme, was significantly up regulated in the Hfe-KO mice compared with the WT mice 8 weeks after surgery (n=5 per group, Figure 4.8).

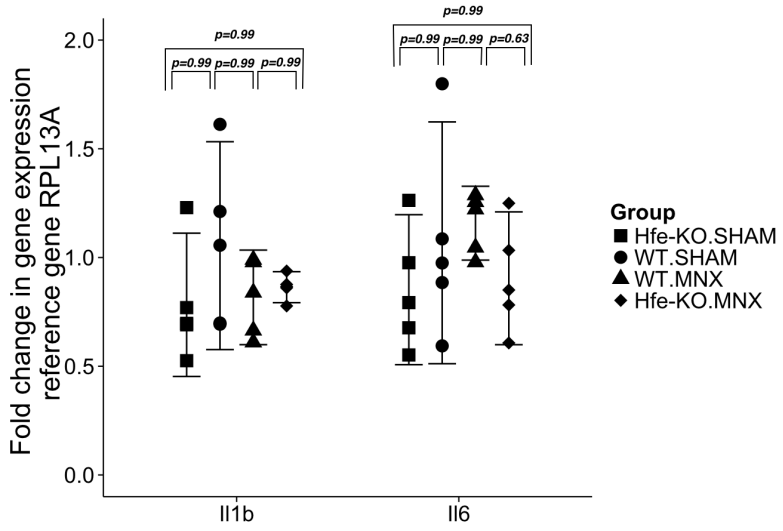

Figure 4.7: Changes in gene expression of pro-inflammatory citokynes following surgical induction of osteoarthritis. Real time PCR, using mRNA isolated from the medial knee compartment of animals euthanized 8 weeks after surgery, to detect the expression of Il-1b and Il-6 in the cartilage and subchondral bone (n=5 per group). There are no significant changes in the expression of these pro-inflammatory cytokines at 8 weeks after surgery since the acute inflammatory response has already subsided and the disease process is in a chronic phase. Data are shown as the individual data points (shapes) with the corresponding 95% CI (range).

In addition, we evaluated the localization of some of the proteins encoded by these genes using immunohistochemical staining. Eight weeks after surgery both the Hfe-KO and the WT MNX-operated knees had a similar percentage of articular chondrocytes positive for type X collagen, ADAMTS-5 and MMP-13, but the Hfe-KO.MNX knees

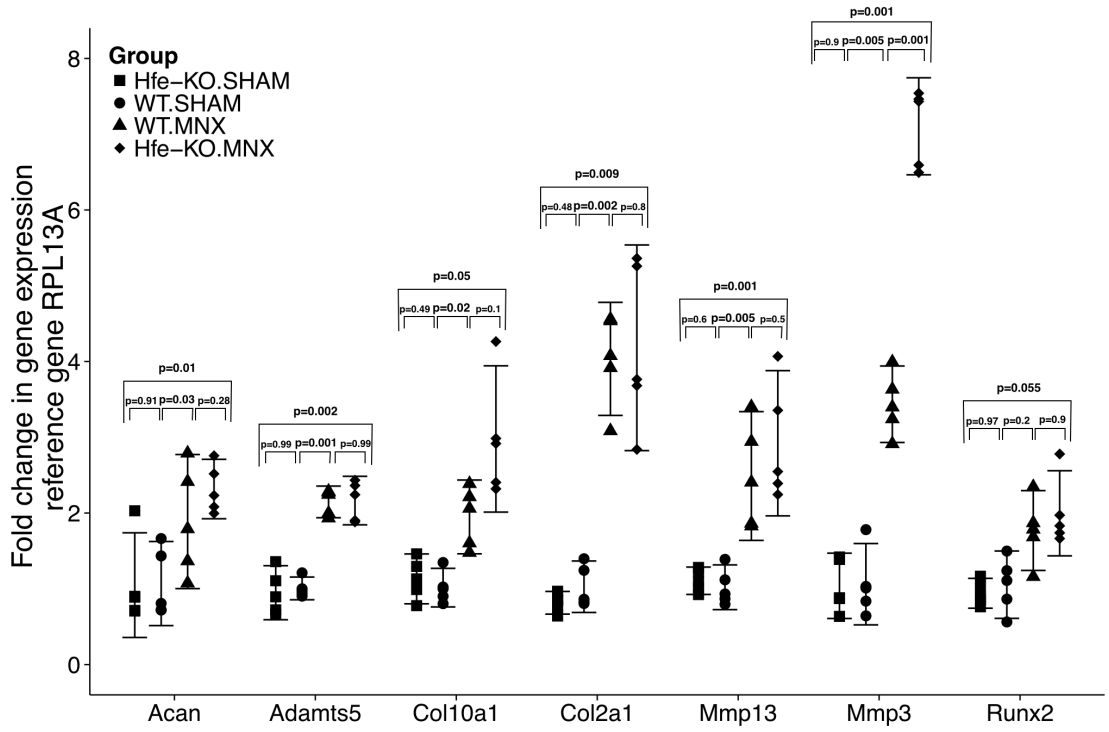

Figure 4.8: Changes in gene expression following surgical induction of osteoarthritis. RT-PCR, using mRNA isolated from the medial knee compartment of animals euthanized 8 weeks after surgery, to detect the expression of selected target genes (n=5 per group). The results confirm that MNX surgery induces the expression of genes related with matrix synthesis, matrix degradation and chondrocyte hypertrophy and that this is more pronounced in the Hfe-KO mice. Data are shown as the individual values (shapes) with the corresponding 95% CI (range).

displayed stronger staining and a significantly greater percentage of MMP-3-positive cells when compared to their controls (n=3 per group, Figure 4.9).

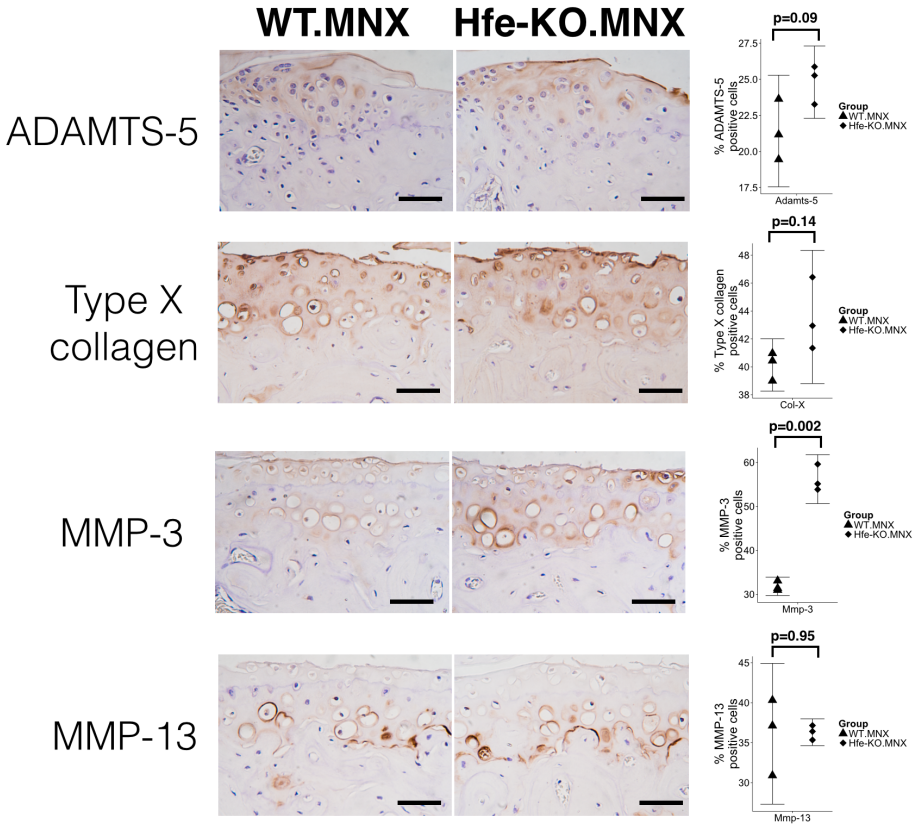

Figure 4.9: Immunohistochemistry for, a disintegrin and metallopeptidase with thrombospondin motifs (ADAMTS-5), type X collagen, matrix metallopeptidase 3 (MMP-3) and matrix metallopeptidase 13 (MMP-13), in the medial tibial plateu at 8 weeks after MNX surgery in control (WT.MNX) and Hfe-KO (Hfe-KO.MNX) mice. 3,3'-diaminobenzidine (DAB) counterstained with Harris haematoxilin, scale bars=50 µm. The ratio of positive cells per field was counted under a microscope at 40x magnification using three sections from three mice. The results show that the levels of MMP-3 enzyme are significantly higher in the Hfe-KO mice knees following MNX surgery. Data are the individual values (shapes) with the corresponding 95% CI (range).

4.2 Paper II

Demographic and clinical characteristics of patients

In total, 306 patients with HH completed the questionnaire. The sample characteristics are shown in Table 4.1. The median age of the patients was 60 years (interquartile range (IQR) = 53 to 68 years); 47.4% were females (80% menopausal). Two hundred and sixty-six patients (87%) were C282Y/C282Y homozygous and 40 (13%) were C282Y/H63D compound heterozygous. Median serum ferritin concentration at diagnosis was significantly higher in patients with C282Y homozygosity [1090 (610 to 2210) $\mu g\,l^{-1}$ vs. 603 (362 to 950) $\mu g\,l^{-1}$, P<0.001] as was median transferrin saturation [80 (66 to 91)% vs. 63 (55 to 72)%, P<0.001]. The proportion of patients with severe iron overload (>1000 $\mu g\,l^{-1}$) was greater among those with C282Y homozygosity (52.4% vs. 20%, P<0.001).

Table 4.1: Characteristics of patients with hereditary hemochromatosis by genotype. Data shown as the median and IQR or percentage. TS: transferrin saturation.

	C282Y/C282Y, n=266	C282Y/H63D, n=40	P value
Age, years	60 (53 to 68)	61 (55 to 67)	0.50
Women, %	48.8	37.5	0.18
Body mass index, $kg\,m^{-2}$	24.8 (22.1 to 26.9)	26.2 (23.5 to 30.3)	0.003
Menopausal women, %	80	80	0.80
Current smoker,%	13.2	7.5	0.30
Serum ferritin concentration at diagnosis, $\mu g\,l^{-1}$	1090 (610 to 2210)	603 (362 to 950)	<0.001
Serum transferrin saturation coefficient, %	80 (66 to 91)	63 (55 to 72)	<0.001

Self-reported symptoms at time of survey

The prevalence of self-reported joint pain at the time of the survey was significantly higher for patients with C282Y homozygosity (88.4% vs. 77.5%, aOR 2.8, 95% CI1.2 - 6.9). The prevalence of self-reported asthenia, diabetes and cardiac disease did not differ between genotypes (Table 4.2).

Table 4.2: Prevalence of self-reported symptoms at time of survey by genotype.OR, odds ratio; aOR, adjusted OR; 95% CI, 95% confidence interval. *Adjusted for age, sex, body mass index and smoking habits.

	C282Y/C282Y, n=266	C282Y/H63D, n= 40	Crude OR (95%CI)	aOR* (95%CI)	P value
Joint pain, %	88.4	77.5	2.2 (0.96-5.1)	2.8 (1.2-6.9)	0.02
Asthenia, %	79.3	82.1	0.82 (0.34-1.9)	0.8 (0.33-1.9)	0.56
Diabetes, %	9.7	10	0.98 (0.32-2.9)	1.2 (0.37-3.9)	0.87
Cardiac disease, %	15.4	17.5	0.86 (0.36-2.1)	0.98 (0.39-2.5)	0.83

OA, joint replacement and spine involvement

The prevalence of OA was higher with C282Y homozygosity than C282Y/H63D compound heterozygosity: 53.4% vs. 32.5% (aOR = 2.4 [95% CI 1.2 – 5.0]; Table 4.3). This difference was no longer significant on adjustment for ferritin concentration (aOR = 1.66 [0.79 – 3.5]). The prevalence of hip replacement was higher for homozygotes than heterozygotes-11.7% vs. 7.5% (aOR = 1.5 [0.41 – 5.1])—but this difference did not reach statistical significance. The prevalence of self-reported back pain and sciatica did not differ between the groups: 71.8% vs. 70% and 47.4% vs. 45%, respectively.

Table 4.3: Prevalence of osteoarthritis, joint replacement, back pain and sciatica by genotype. OR, odds ratio; aOR, adjusted OR; 95% CI, 95% confidence interval. *Adjusted for age, sex and body mass index.

	C282Y/C282Y, n=266	C282Y/H63D, n= 40	Crude OR (95%CI)	aOR* (95%CI)	P value
Osteoarthritis, %	53.4	32.5	2.4 (1.2-4.8)	2.4 (1.2-5)	0.02
Knee replacement, %	5.6	5.0	1.1 (0.25-5.1)	1.5 (0.3-7.1)	0.64
Hip replacement, %	11.7	7.5	1.6 (0.47-5.6)	1.5(0.41-5.1)	0.08
Ankle replacement, %	0.75	2.5	0.3 (0.03-3.3)	0.32 (0.03-4)	0.52
Back pain, %	71.8	70	1.1 (0.53-2.3)	1.2 (0.55-2.6)	0.31
Sciatica, %	47.4	45	1.1 (0.56-2.1)	1.3 (0.65-2.6)	0.59

Osteoporosis and fractures by genotype

In total, 155 patients (50.7%) reported that they had undergone dual-energy X-ray absorptiometry (DEXA). All the patients who self-reported OP also reported having undergone DEXA. The prevalence of OP was greater for C282Y homozygous than compound heterozygous patients (25.6% vs. 7.5%; aOR = 3.5 [95% CI 1.1–12.1]); Table 4.4). The effect of genotype on the prevalence of OP was no longer statistically significant on adjustment for ferritin concentration (aOR = 2.6 [0.74 – 9.3]).

The prevalence of hip, wrist and vertebral fractures did not differ between the groups: 2.6% vs. 2.5%, 13.2% vs. 7.5% and 7.9% vs. 2.5%, respectively.

Table 4.4: Comparison of the prevalence of osteoporosis and associated fractures by genotype. OR, odds ratio; aOR, adjusted OR; 95% CI, 95% confidence interval. *Adjusted for age, sex, body mass index, menopausal status and smoking habits.

	C282Y/C282Y, n=266	C282Y/H63D, n= 40	Crude OR (95%CI)	aOR* (95%CI)	P value
Osteoporosis, %	25.6	7.5	4.3 (1.3-14.2)	3.5 (1.1-12.1)	0.04
Hip fracture, %	2.6	2.5	1.1(0.13-8.8)	0.66 (0.07-6.3)	0.64
Wrist fracture, %	13.2	7.5	1.9 (0.55-6.4)	2.3 (0.65-8.2)	0.45
Vertebral fracture, %	7.9	2.5	3.3 (0.44-25.6)	3.3 (0.42-26)	0.15

Chapter 5

Discussion

Systemic iron overload secondary to hemochromatosis is associated with articular cartilage degeneration, and increased prevalence of osteoarthritis (Husar-Memmer *et al.*, 2014).

In paper I, experimental OA was surgically induced in the knee of a mouse model of hereditary hemochromatosis. The knee joints were then evaluated to determine articular cartilage degeneration, subchondral bone changes and gene expression patterns. In paper II a large cohort of patients with hereditary hemochromatosis was evaluated in order to determine the effect of HH genotype on patients self-reported musculoskeletal complications.

5.0.1 Limitations of the study

The study described in paper I has some limitations; in humans, HH-related OA develops spontaneously in the fourth and fifth decades of life whereas we used young mice (10-weeks-old) to study the development of surgically induced OA. Therefore this model may not accurately represent the progression of the human disease. In addition, since the observed changes in cartilage and subchondral bone were restricted to a single time-point it is difficult to conclude about the entire sequence of genetic and morphologic changes leading to OA, but this could be addressed in future studies. The 8-week time point was selected since both cartilage and subchondral bone changes are already well defined (Kamekura *et al.*, 2005) and the disease process is in a quiescent phase (Loeser *et al.*, 2013).

This study addresses the changes in a limited number of target genes, whose selection was geared towards the main components of the extracellular matrix (Martin *et al.*, 2001), the main proteolytic enzymes involved in matrix degradation (Reboul *et al.*, 1996; Blom *et al.*, 2007; Verma & Dalal, 2011), and the main molecules related with chondrocyte hypertrophy (Zheng *et al.*, 2003). However, it is possible that other genes play a relevant role in the development of HH-related OA, a possibility that could be addressed in future studies.

The study described in paper II also has limitations; patients were asked to remember

symptoms attributed to hemochromatosis, which might have induced recall bias (Pannucci & Wilkins, 2010). Because of the relatively low number of patients with compound heterozygosity, the number of rare events such as fractures or joint replacement surgery was low in this group. This might explain the non-significant findings for joint replacement and fracture.

5.0.2 Changes in cartilage and bone in iron overloaded joints

As previously described (Levy *et al.*, 1999), the Hfe-KO mutation of our mouse model, a deletion of a large portion of the Hfe gene coding sequence generating a null allele, had no effect on the morphology of the mice, but caused a significant systemic iron overload with increased iron content observed in the serum and liver of these mice.

Following arthrotomy, the Hfe-KO mice showed an increased deposition of iron in the form of hemosiderin in the synovial membrane. Synovial iron accumulation can also be found in human HH patients and in patients with rheumatoid arthritis (RA) (Kra *et al.*, 1965; Walker *et al.*, 1972; Heiland *et al.*, 2010). It is thought that the iron arises not from exchange with the labile iron pool but from blood that enters the joint, either by trauma or by oozing from an inflamed synovial membrane (Muirden & Senator, 1968). Accordingly, we hypothesise that the iron seen in the knee synovial membrane would originate from blood entering the joint following the arthrotomy. The greater hemosiderin accumulation in the Hfe-KO mice joints could, therefore, be explained by the higher iron content of their blood.

On its own, iron accumulation in the synovial membrane does not appear to be enough to cause cartilage damage in this time period, as shown by the data from Hfe-KO mice SHAM-operated knees, since their histological, morphological and gene expression characteristics do not show any evidence of OA and are identical to their WT counterparts. Feeding the mice with an iron-enriched diet in order to obtain an even greater iron overload or having a longer follow-up period could yield different results.

The precise role of subchondral bone in the initiation and progression of OA is still unclear (Radin & Rose, 1986; Oegema *et al.*, 1997; Burr, 2004). A recent review (Li *et al.*, 2013), proposes that the non-physiologic strain on the joint, secondary to mechanical overload caused by either joint instability or reduced congruency, causes micro-damage to the subchondral bone. This could then lead to increased subchondral bone resorption and a decrease in thickness in the early-stages of OA. The process of subchondral bone remodelling progresses to subchondral trabecular sclerosis and increased calcified cartilage thickness in the late-stage OA. These changes in subchondral bone architecture are likely to accompany cartilage damage that could, in turn, influence subchondral bone degradation, resulting in a vicious cycle. In line with this hypothesis, at 8 weeks post-surgery, the MNX-operated knees showed features of late-stage OA, namely increased subchondral bone volume and calcified cartilage thickness. These changes were more pronounced in the joints with more severe articular cartilage damage, a finding which was also congruent with data previously reported by other authors (Chappard *et al.*, 2006; Hayami *et al.*, 2006; Botter *et al.*, 2009).

5.0.3 Genetic expression in iron overloaded joints

As previously reported in the literature, we found that surgically induced mechanical instability of the knee joint resulted in deregulation of genes related to extracellular matrix production, matrix degradation and chondrocyte hypertrophy (Appleton *et al.*, 2007; Loeser *et al.*, 2013; Bateman *et al.*, 2013). These changes were observable in both WT and Hfe-KO mice meniscectomized knees, but differences were more evident in the latter group, providing further evidence for the increased propensity to OA in the Hfe-KO mice.

The up-regulation of Acan and Col2a1 present in the MNX knees of both strains probably represents an anabolic response to the cartilage damage. Ultimately it fails to repair the damaged cartilage, since the mechanical overload is sustained and induces an intense catabolic response as shown by the up-regulation of matrix proteases.

The presence of excess iron in the synovial tissue could account for the accelerated OA progression in the meniscectomized knees of the Hfe-KO mice since intra- and extracellular overload of iron in the tissue contributes to an increased generation of ROS after an initial pro-inflamatory signal (Morris *et al.*, 1986; Sullivan, 2004; Brissot *et al.*, 2012), such as joint trauma or surgical intervention. The ROS can lead to hyper-activation of MMPs (Gurjar *et al.*, 2001; Zamboni *et al.*, 2005; Mairuae *et al.*, 2011), a family of enzymes involved in the breakdown of the extracellular matrix, which is a crucial step in many normal as well as pathological biological processes (Nagase & Woessner, 1999). Specifically, recent studies show that the overproduction of ROS in the mitochondria of the chondrocytes leads to an up-regulation of MMP genes (Reed *et al.*, 2014) and to increased chondrocyte apoptosis (Collins *et al.*, 2015), contributing to articular cartilage damage.

Consistent with this hypothesis, following the surgical induction of OA, the Hfe-KO mice displayed an increased expression of the Mmp3 gene and increased levels of the enzyme, when compared to their WT controls. This enzyme is capable of degrading several components of the extracellular matrix (Hasty *et al.*, 1990; Wu *et al.*, 1991), making it less resilient to the mechanical stress and accelerating the progression of OA (Blom *et al.*, 2007; Bateman *et al.*, 2013). Therefore an increase in Mmp3 expression could be one of the factors contributing to the greater susceptibility to cartilage damage observed in the Hfe-KO mice knees.

The increase in Runx2 expression found on MNX-operated knees was related to the mechanical overload caused by the meniscectomy. Previous studies found that mechanical overload on the joints leads to induction of the transcription activator Runx2 (Kawaguchi, 2008), increasing the expression of type X collagen (Zheng *et al.*, 2003), which in turn will lead to chondrocyte hypertrophy, apoptosis and endochondral ossification. In accordance with previous studies (von der Mark *et al.*, 1992; Eerola *et al.*, 1998), the expression of type X collagen was altered in the joints presenting OA-related morphological changes and an increase in type X collagen expression was positively correlated to the severity of cartilage degeneration. These findings are in agreement with previous data from Walker et al (Walker *et al.*, 1995), who have shown that expression of type X collagen was induced in response to the extracellular matrix damage caused by the osteoarthritic

process. The increased expression of Bmp6 in the MNX-operated joints of Hfe-KO mice could be secondary to the iron overload, since the BMP6-SMAD signalling pathway is an important regulator of hepcidin expression (Andriopoulos *et al.*, 2009; Corradini *et al.*, 2009; Corradini *et al.*, 2011). Bmp6 also has an anabolic role in bone metabolism since it is expressed in hypertrophic chondrocytes, inducing the formation of endochondral bone (Kugimiya *et al.*, 2005). This data is coherent with our findings in that the increased expression of Bmp6 was observed in the knees with greater subchondral bone thickness and with greater type X collagen expression.

Previous reports show that the levels of proinflammatory cytokines are elevated in the first days following injury (Irie *et al.*, 2003; Haslauer *et al.*, 2014) and then gradually decrease in the following weeks, eventually returning to normal levels (Bigoni *et al.*, 2013; Ward *et al.*, 2008). The absence of significant differences in the expression of Il1b and Il6 in this study could be explained by the fact that the mice were evaluated at 8 weeks after the initial injury, when the disease process is already in a quiescent phase (Loeser *et al.*, 2013).

5.0.4 Role of genotype in musculoskeletal complications

In accordance with previous reports (Pawlotsky *et al.*, 1999; Burke *et al.*, 2000; Walsh *et al.*, 2006; Saliou *et al.*, 2013) , the HH patients in paper II, with the homozygous C282Y/C282Y genotype, showed significantly higher ferritin and transferrin saturation coefficient values, when compared to patients with compound heterozygosity. Interestingly, about half of the homozygous patients showed severe iron overload. BMI was greater for compound heterozygous than for homozygous patients, similarly to what was found recently by other workers (Wang *et al.*, 2012; Saliou *et al.*, 2013). Indeed, comorbid factors such as alcohol abuse and obesity are more prevalent in C282Y/H63D than C282Y/C282Y patients (Saliou *et al.*, 2013). The two genotype groups did not differ in prevalence of self-reported asthenia, cardiac disease or diabetes, findings which are similar to those reported by Waalen et al. (Waalen *et al.*, 2002), showing that patients with different genotypes of HFE self-reported similar rates of symptoms commonly associated with iron overload.

Several studies (Olynyk *et al.*, 1999; Bulaj *et al.*, 2000; von Kempis, 2001; Ross *et al.*, 2003; Cauza *et al.*, 2005; Carroll, 2006; Alizadeh *et al.*, 2007; Allen *et al.*, 2008; Crooks *et al.*, 2009; Sahinbegovic *et al.*, 2010b; Zwerina & Dallos, 2010) have documented the association of HH with an clinically relevant arthropathy that may seriously affect quality of life (Adams & Speechley, 1996). This arthropathy can be severe, as highlighted by the high prevalence of hip, knee and ankle joint replacement in these patients (Richette *et al.*, 2010; Sahinbegovic *et al.*, 2010a; Elmberg *et al.*, 2013).

In our study, prevalence of self-reported OA was greater in patients with C282Y homozygosity than C282Y/H63D compound heterozygosity, after adjustment for confounding variables. The prevalence of hip replacement surgery had a tendency to be increased, among patients with C282Y homozygosity, when compared to patients with compound heterozygosity, as reported recently by Wang (Wang *et al.*, 2012). This increased prevalence of OA in C282Y homozygotes might be related to the more severe

iron overload in these patients.

Indeed, although the role of iron overload in the genesis of HH-related OA is still not fully understood (Husar-Memmer *et al.*, 2014), it can lead to iron accumulation in the synovium and cartilage of HH patients, as seen in histological studies (Walker *et al.*, 1972; Schumacher, 1982), and to increased ferritin concentrations in synovial fluid (Carroll *et al.*, 2010). Some of this iron may be in the form of non-transferrin–bound iron (de Valk *et al.*, 2000), and might therefore have deleterious effects (Brissot *et al.*, 2012) on synovial tissue, promoting inflammation due to neutrophil infiltration (Heiland *et al.*, 2010) and tissue damage (Pantopoulos *et al.*, 2012), as was demonstrated for the liver (George *et al.*, 1998). Iron deposits were found in synovial tissue from patients with inflammatory arthritis (Muirden & Senator, 1968), which suggests a link between iron content and inflammation. More recently, the serum level of vascular adhesion molecule 1 (VCAM-1) was found associated with radiographic measures of HH arthropathy, which suggests its involvement in HH joint damage (Nell-Duxneuner *et al.*, 2013).

Patients with HH have shown disc degeneration and calcifications in the intervertebral discs due to calcium pyrophosphate crystal deposits (Bywaters *et al.*, 1971; Hamilton *et al.*, 1981). In a retrospective study (Valenti *et al.*, 2008), one third of patients with HH showed spine arthropathy on X-ray. The literature contains no data on the prevalence of low back pain in patients with a clinical phenotype of HH. Both our genotype groups showed similar self-reported prevalence of low back pain and sciatica, which suggests that the lumbar spine involvement in HH is independent of the genotype.

We found an increased prevalence of OP, along with increased prevalence, (although not statistically significant), of vertebral fractures in homozygote patients. Again, this observation could be related to the increased iron overload observed in C282Y homozygotes. Indeed, several reports (Guggenbuhl *et al.*, 2005; Weinberg, 2008) have shown that HH patients, particularly males, are at increased risk of OP, which might be related to iron overload (Valenti *et al.*, 2009). The pathophysiology of OP in HH is not entirely clear. Iron-overloaded mice show increased levels of reactive oxygen species, elevated serum TNF-α and Il6, which are known to be associated with the severity of iron overload (Tsay *et al.*, 2010). HFE-/- mice show impaired bone microarchitecture and increased osteoclast number (Guggenbuhl *et al.*, 2011a). In vitro, iron disrupts the formation of hydroxyapatite crystals (Guggenbuhl *et al.*, 2008) and inhibits the proliferation and differentiation of osteoblasts (Messer *et al.*, 2009; Yang *et al.*, 2011). As reported by the previous studies, animal and in vitro data support the hypothesis that iron in excess can directly affect bone formation and remodeling (de Vernejoul *et al.*, 1984; Isomura *et al.*, 2004) contributing to the onset of OP.

5.0.5 Future directions

One interesting question to be addressed in a future study is the pattern of gene expression in the synovial membrane in the presence of iron overload. The synovial cells produce inflammatory mediators that lead to cartilage breakdown (Sellam & Berenbaum, 2010) and also have the ability to produce ROS with tissue damaging potential in the presence of iron (Morris *et al.*, 1986). Also, a recent study (Lambert *et al.*, 2014) showed that in

the synovial membrane of patients undergoing joint replacement surgery, there was an up-regulation of the expression of the BMP6 gene, among others. BMP6 is one of the key regulators of iron metabolism (Meynard *et al.*, 2009) the up-regulation of this gene in the synovial membrane of end-stage OA could mean that iron overload also plays a role in the development of idiopathic OA.

Another interesting follow-up study would be to investigate the effect of the Mmp3-KO mutation (Li *et al.*, 2004) in the Hfe-KO mice. In addition, it will be important to know if there are other proteolytic enzymes with a relevant role in cartilage degradation beside the ones already studied here. A whole transcriptome sequencing approach coupled to a proteome identification strategy could be of relevance to provide further answers to this important question.

Alternatively, it would be interesting to perform a microarray study of cartilage and synovial tissue from HH patients with and without arthropathy. The objective would be to identify differentially expressed genes in the healthy and diseased joints of patients with clinical HH. Hopefully these genes could act as makers of OA progression and be used to identify patients at risk of developing HH-related OA. This information could also contribute to find possible therapeutic targets for OA, a result that would be very relevant given the large number of patients worldwide, with a tendency to increase, due to the expected ageing of the population.

Chapter 6

Conclusions

At 18 weeks, mice with the Hfe-KO phenotype had a systemic iron overload showing higher iron content in liver, plasma and synovial membrane when compared to their WT controls.

Meniscectomy caused degradation of articular cartilage, sclerosis of the subchondral bone and increased expression of genes linked with ECM synthesis, chondrocyte hypertrophy and matrix proteases.

The gene expression profile observed in the mice is compatible with a remodelling response, up-regulation of the anabolic pathway, increasing the formation of ECM in order to repair the damaged areas. This response is not effective, since the sustained adverse mechanical environment induces the catabolic pathway, with the production of matrix-degrading enzymes, resulting in the degradation of the hyaline articular cartilage and increased chondrocyte hypertrophy.

At the same time, the osteogenic pathway observed in the growth plate is reinstated and the articular calcified cartilage undergoes ossification leading to an increased thickness of the subchondral bone plate. The trabeculae of the subchondral zone also experienced an abnormal growth. This abnormally thickened and sclerotic bone is mainly located in the zones of greater cartilage destruction.

All the changes stated above, particularly the expression of the gene encoding Mmp3, were more pronounced in the Hfe-KO animals resulting in a more severe phenotype following the surgical induction of OA.

Although there was a visible hemosiderin accumulation in the SHAM operated joints of the Hfe-KO mice, it was not enough to cause cartilage degradation or subchondral bone changes at eight weeks post-surgery.

The findings stated above suggest that systemic iron overload is not a direct cause of OA, but acts as a susceptibility factor, amplifying the damage caused by mechanical overload of the joint.

In humans with hereditary hemochromatosis, homozygosity for the C282Y mutation was associated with a more severe iron overload, when compared with compound heterozygotes C282Y/H63D. The homozygotes also had an increased prevalence of musculoskeletal HH-related complications suggesting a relationship between the systemic iron

overload and the damage to bone and cartilage.

Our findings in mice could help to explain why HH-related OA does not affect all patients that have clinical manifestations of HH, and usually is only symptomatic at the fourth and fifth decades of life. According to our model, not only would the patient have to develop a clinically relevant iron overload, but also to sustain some form of damage to their joints, probably in the form of minor traumas that are common in everyday life. Such traumatic episodes typically cause oozing of blood into the joint. Since HH patients usually have high blood iron concentrations, the amount of iron entering the joint and being deposited in the synovial membrane in the form of hemosiderin would be considerable. After several episodes of minor trauma there might be enough iron accumulation in the synovial membrane to act as a susceptibility factor, amplifying the deleterious effect of subtle mechanical overload of the joints, and leading to an accelerated OA progression in these patients.

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Appendix A

Paper I

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Osteoarthritis and Cartilage

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Iron overload in a murine model of hereditary hemochromatosis is associated with accelerated progression of osteoarthritis under mechanical stress

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SUMMARY

Objective: Hereditary hemochromatosis (HH) is a disease caused by mutations in the *Hfe* gene characterised by systemic iron overload and associated with an increased prevalence of osteoarthritis (OA) but the role of iron overload in the development of OA is still undefined. To further understand the molecular mechanisms involved we have used a murine model of HH and studied the progression of experimental OA under mechanical stress.

Design: OA was surgically induced in the knee joints of 10-week-old C57BL6 (wild-type) mice and *Hfe*-KO mice. OA progression was assessed using histology, micro CT, gene expression and immunohistochemistry at 8 weeks after surgery.

Results: *Hfe*-KO mice showed a systemic iron overload and an increased iron accumulation in the knee synovial membrane following surgery. The histological OA score was significantly higher in the *Hfe*-KO mice at 8 weeks after surgery. Micro CT study of the proximal tibia revealed increased subchondral bone volume and increased trabecular thickness. Gene expression and immunohistochemical analysis showed a significant increase in the expression of matrix metallopeptidase 3 (MMP-3) in the joints of *Hfe*-KO mice compared with control mice at 8 weeks after surgery.

Conclusions: HH was associated with an accelerated development of OA in mice. Our findings suggest that synovial iron overload has a definite role in the progression of HH-related OA.

Introduction

Osteoarthritis (OA) is a disease of the joints characterized by progressive articular cartilage destruction and subchondral bone changes. Several factors influence OA development, including genetic background, past joint injuries, mechanical overload and ageing[1,2]. The pathologic process that leads to cartilage destruction involves a disruption of the normal resting state of chondrocytes, leading to an increased production of both matrix proteins and matrix degrading enzymes such as matrix metallopeptidase (MMP) and a disintegrin and metallopeptidase with thrombospondin motifs (ADAMTS)[3].

Hereditary hemochromatosis (HH), a chronic disease caused by mutations in the *Hfe* gene[4] and characterized by systemic iron overload that causes damage in the liver, heart and endocrine organs[5], is associated with an increased incidence of OA[6] and joint replacement surgery[7–9]. Although liver and heart complications are the main causes of mortality in patients with HH, arthropathy has the greatest impact on the quality of life and rarely benefits

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from therapeutic phlebotomies[10]. Studies of human joint tissue have shown hemosiderin accumulation in the cartilage and synovial tissue and histological features reminiscent of OA and of rheumatoid arthritis (RA)[11,12] and increased ferritin levels in the synovial fluid[13] of affected joints, but it is unclear how the iron overload damages the joints and which HH patients will develop musculoskeletal complications[14].

Previous reports have shown that murine models of HH can be useful in studying the pathogenesis of HH-related liver disease[15], HH-related diabetes[16] and HH-related changes in bone metabolism[17], but there are no studies using a murine model of HH to investigate the effect of iron overload in articular cartilage.

In order to investigate the role of iron overload in the development of OA we induced OA[18] in a mouse model of HH[19] and studied the subsequent morphological, histological and genetic changes occurring in the cartilage and subchondral bone of the knee joint.

Materials and methods

Details of additional data, methods, primers and reagents are available in the Supplementary information material.

Animal model, experimental procedure, feeding and housing

The *Hfe*-KO mice in a C57BL/6 background[19] (HFE-KO) were used as a model for human HH. C57Bl/6 mice from the University of Algarve Animal Facility in-house colony were used as wild-type controls. Distribution of animals between groups and tasks is further explained in Supplementary fig. 1. Animals were maintained in specific pathogen-free conditions and had access to water and food *ad-libitum* from weaning up to time of euthanasia. OA was induced in the right knee joint of 10-week-old Hfe-KO and wild-type mice by sectioning the medial collateral ligament and excising of the medial meniscus (MNX) using a microscope[18]. The left knee was sham-operated (SHAM). All the animals were euthanized 8 weeks after surgery and bilateral knee joints were collected, processed and used as described below. All the procedures were approved by the Portuguese National Authority for Animal Health (Ref. 0421/000/000/2013) and by the Animal Facility of University of Algarve.

Assessment of iron accumulation

For the assessment of iron accumulation in the liver and blood, samples of six animals of each strain were used. At the time of euthanasia blood was collected by intra-cardiac puncture for determination of serum iron, serum ferritin and serum transferrin saturation; liver tissue was collected to determine the hepatic non-heme iron concentration[20].

Grading of osteoarthritic changes

Bilateral knee joints from ten animals in each group were isolated, cleaned of adherent soft tissues in ice-cold PBS and fixed for 24 hrs in 4% paraformaldehyde in PBS pH 7.4, followed by decalcification with 0.5 M Ethylenedinitrilotetraacetic acid (EDTA) in PBS pH 7.4 for 3 weeks. After dehydration through graded alcohols and inclusion in paraffin, 5 μm sagittal sections were cut from the medial compartment of the joints and stained with Safranin-O/Fast Green/Meyer's Hematoxylin. Two separate observers, blinded to the strain and intervention, graded the cartilage lesions (grade 0–6) using the Osteoarthritis Research Society International (OARSI) scoring system for murine OA[21]. The medial tibial plateau and the medial femoral condyle were graded separately. The final score for each joint was the average of both observers score.

Morphological characterization of the knee joint by micro-CT

Micro-CT was performed on bilateral knee joints of five animals in each group with a Skyscan 1172 X-ray computed microtomograph (Bruker, Belgium). For the image acquisition the following parameters were used: X-ray tube potential 70 kVp, X-ray tube current 100 μA, 0.5 mm Al filter, rotation step 0.4°, isotropic voxel size 5 μm^3, integration time 500 ms, frame averaging = 6. The proximal epiphysis of the tibia was selected as region of interest (ROI). To quantify the characteristics of the subchondral bone[22] the epiphysis was further divided in a cortical part (subchondral bone plate) and a trabecular part. The following 3D morphometric parameters were used to describe the bone of the trabecular compartment: Bone volume fraction (BV/TV, in %) is the ratio of the segmented bone volume to the total volume of the region of interest; Connective density (Conn.D, in mm^{-3}) is a measure of the average number of trabeculae per unit of volume; Trabecular thickness (Tb.Th, in μm) is the mean thickness of trabeculae. To describe the subchondral bone plate we measured the average thickness (Sb.Th, in μm).

Histological evaluation of undecalcified samples

Following micro-CT the samples were included in methyl methacrylate (MMA) at 4°C. From these undecalcified samples, 5 μm sagittal sections were cut from the medial knee compartment on a heavy-duty microtome equipped with tungsten carbide knives. Sections were stained with Perl's and counterstained with Neutral Red in order to assess iron deposition. The regions of interest for this analysis were the knee joint and the proximal epiphysis of the tibia. The total area of hemosiderin deposits in the synovial membrane was measured as previously described[11].

Evaluation of gene expression

The bilateral knee joints of ten mice from each strain were used for RNA isolation. Immediately after euthanasia both knee joints were immersed in RNALater at 4°C and cleaned of soft tissues, in order to isolate the tibial and femoral cartilage and subchondral bone of the medial knee compartment. RNA was extracted using TRI Reagent (Sigma–Aldrich), purified using the High Pure RNA Isolation Kit (Roche) and quantified with an Experion RNA analysis system (Bio-Rad). The reverse transcription reaction (RT) was carried out using 0.5 μg of total RNA per reaction with the M-MLV reverse transcriptase (Invitrogen) according to the manufacturer indications. Real-time PCR was carried out in a Bio-Rad CFX-96 machine, using the parameters specified by the manufacturer for the combination of machine and master mix. The *Rpl13a* gene was used as control of endogenous gene expression[23] and the WT.SHAM group as the reference condition.

Immunohistochemistry

Bilateral knee joints of three mice of each strain were used for immunohistochemical localization. After dewaxing and rehydration, epitope retrieval was performed and the sections were incubated with polyclonal rabbit antibodies against mouse MMP-3, MMP-13 (Proteintech, England), ADAMTS-5 (Abcam ab41037) at a dilution of 1:40 and against type X collagen (Abcam ab58632) at a dilution of 1:20 for 24 h at 4°C. The sections were then treated with 0.3% hydrogen peroxide to block endogenous peroxidases, incubated with horseradish peroxidase-conjugated goat antibodies against rabbit IgG (Sigma–Aldrich) at a 1:100 dilution. After rinsing, the sections were incubated with a 0.05% 3,3′-diaminobenzidine solution to visualize the location of the target

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protein-primary-secondary antibodies complex and counter-stained with Mayer's haematoxylin.

Statistical analysis

Unless stated otherwise, results are expressed as the mean and corresponding 95% Confidence Interval (95% CI). The Student's-*t* test was used to compare continuous variables between different strains. The Mann-Whitney *U* test was used when comparing ordinal variables or when the assumptions for a parametric test were not met. Pairwise comparisons using paired *t*-tests with Holm's *P*-value adjustment were used to compare continuous variables between different groups, in order to account for the correlation between joints of the same animal. The Wilcoxon paired test with Holm's *P*-value adjustment was used for ordinal variables. A two-tailed *P*-value < 0.05 was considered statistically significant. All analysis and plotting were conducted using the R v3.1.1 (R Foundation for Statistical Computing, Vienna, Austria) software.

Results

All the animals initially operated were included in the final analysis; the experimental procedure had no adverse effects on the mice.

Mice not expressing the Hfe allele have increased iron accumulation in the serum, liver and knee synovial membrane

The Hfe-KO mice had a plasmatic iron overload ($n = 6$ per group, Fig. 1(A)) and up to five fold increase in hepatic iron content when compared to their controls [1121, 95% CI (927, 1316) μg iron/mg of wet liver weight vs 282, 95% CI (196, 367) μg iron/mg of wet liver weight; *P*-value = 0.002, respectively, $n = 6$ per group].

To evaluate the iron accumulation in the joints of Hfe-KO mice we used undecalcified, MMA-embedded sections of the knee, stained with Perl's. We observed hemosiderin deposits in the synovial membrane of the knee but not in the cartilage (Supplementary fig. 2). These occupied a greater area in the Hfe-KO mice, although there was no significant difference between the MNX and the SHAM operated sides ($n = 5$ per group, Fig. 1(B)). Unpublished data from our research team (Simão M) showed that in non-operated knees of Hfe-KO mice there is no accumulation of hemosiderin in the synovial membrane at 18 weeks of age (Supplementary fig. 3). These observations suggest that the hemosiderin deposits originate from the blood that enters the joint following the arthrotomy. The higher iron content of the Hfe-KO mice blood could explain the greater hemosiderin accumulation.

Using RNA extracted from the subchondral bone and articular cartilage we examined the *Hfe* and *Tfrc* mRNA expression. Real-time PCR analysis confirmed the decreased *Hfe* expression in the Hfe-KO

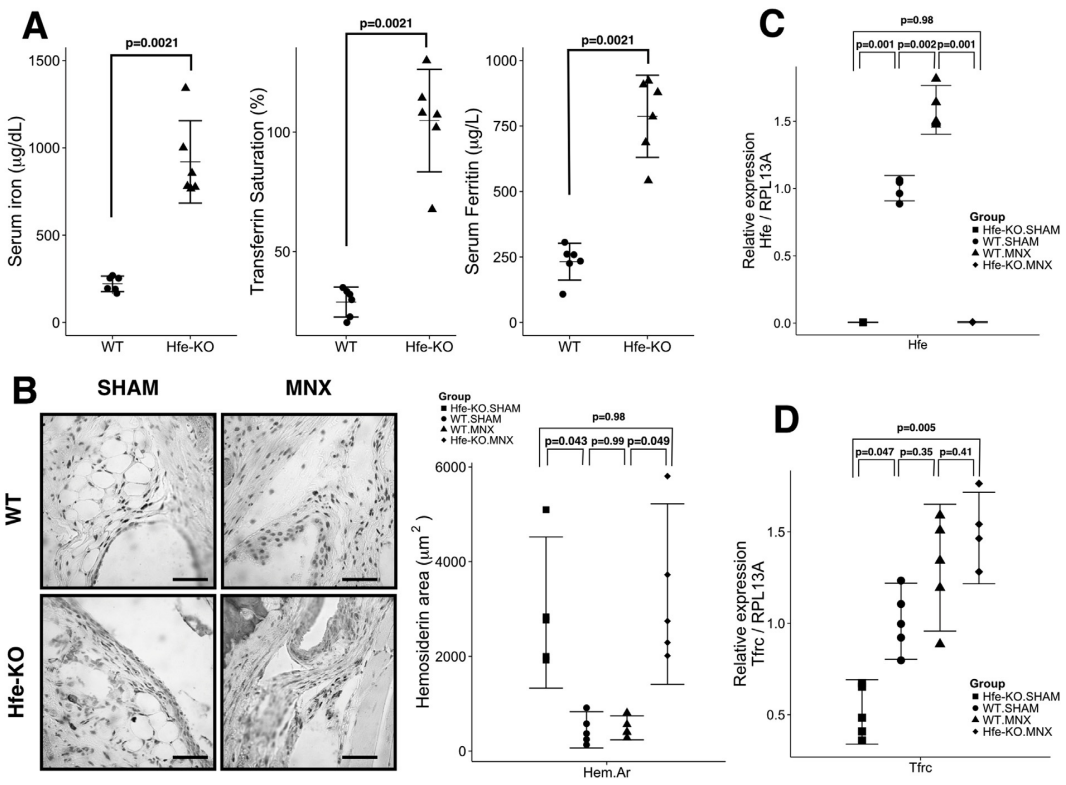

Fig. 1. Iron parameters in WT and Hfe-KO mice (A) Bar plots for the different serum iron parameters. (B) Perl's staining of MMA-embedded knee joint, high magnification view of the knee synovial membrane to observe the hemosiderin deposits (blue) and the quantification of their area showing a greater accumulation of iron in the synovial membrane of the Hfe-KO mice. Scale bar 50 μm. (C) Real time PCR, using mRNA isolated from the medial knee compartment of 18 weeks-old animals, to detect the expression of the *Hfe* gene in the Hfe-KO and control animals ($n = 5$ per group). The results confirm that the *Hfe* gene is not expressed in the bone and cartilage of the Hfe-KO mice. (D) Real time PCR, using mRNA isolated from the medial knee compartment of 18 weeks-old animals, to detect the expression of the *Tfrc* gene. The results show that in Hfe-KO there is an underexpression of the *Tfrc* gene and that the medial meniscectomy leads to an overexpression of this gene. Data are shown as the individual values (shapes) with the corresponding 95% CI (range).

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mice ($n = 5$ per group, Fig. 1(C)). The *Tfrc* expression was decreased in the Hfe-KO SHAM-operated knees and was elevated in both strains on the MNX-operated knees. ($n = 5$ per group, Fig. 1(D)).

Hfe-KO mice have increased cartilage degeneration and subchondral bone volume following the surgical induction of OA

The Hfe-KO mice were morphologically indistinguishable from their WT counterparts and had similar body weight at the time of euthanasia [24.5, 95% CI (23.4, 25.6) g vs 25.2, 95% CI (24.3, 26.1) g respectively; *P*-value = 0.33; $n = 28$ per strain].

To evaluate the effect of the iron overload in the development of OA we surgically induced OA in Hfe-KO mice and in WT controls, using a previously described technique[18]. At 8 weeks after the intervention the MNX-operated knees of the Hfe-KO mice showed a higher level of cartilage destruction [Fig. 2(A)], resulting in a higher summed tibial and femoral OARSI scores for the medial femoral condyle and medial tibial plateau ($n = 10$ per group, Fig. 2(B)). We assessed the articular calcified cartilage (ACC) and the hyaline articular cartilage (HAC) of the tibial plateau in the undecalcified, MMA-embedded knee sections. The MNX operated knees had a lower ACC thickness, lower HAC/ACC ratio and greater subchondral bone height ($n = 5$ per group, Supplementary fig. 4).

To better evaluate the subchondral bone architecture of the medial tibial plateau, we performed micro-CT scans of the knee joints [Fig. 3(A)]. The MNX-operated knees in both strains showed an increase in bone volume, in trabecular thickness and in subchondral plate thickness, but these increases were greater in the Hfe-KO mice ($n = 5$ per group, Fig. 3(B)).

Using RNA extracted from the subchondral bone and articular cartilage we examined *Bmp6* mRNA expression, a signalling molecule involved in iron homeostasis[24] and bone formation[25] and found it to be increased in the Hfe-KO MNX operated knees compared to their WT controls ($n = 5$ per group, Fig. 3(C)). These findings suggest that the Hfe-KO animals have an accelerated progression of the experimental OA, when compared to their WT controls.

The expression of a gene related to cartilage degradation is increased in knee joints of the Hfe-KO mice

To have a better understanding of the accelerated progression of the articular damage in the Hfe-KO mice, we investigated the expression of genes known to be involved in the acute inflammatory response and in the development of OA, namely genes coding for components of the extracellular matrix, chondrocyte hypertrophic markers, factors that promote bone formation, cartilage-degrading enzymes and pro-inflammatory cytokines.

Although cytokines Il1b and Il6 showed no meaningful change in expression at 8-weeks post surgery (Supplementary fig. 5), the remaining genes were up regulated in all MNX-operated knees ($n = 5$ per group, Fig. 4). Only *Mmp3*, a cartilage-degrading enzyme, was up regulated in the Hfe-KO, MNX operated knees when compared with their WT.MNX controls, at 8 weeks after surgery ($n = 5$ per group, Fig. 4).

In addition, we evaluated the localization of some of the proteins encoded by these genes using immunohistochemical staining. Eight weeks after surgery both the Hfe-KO and the WT MNX

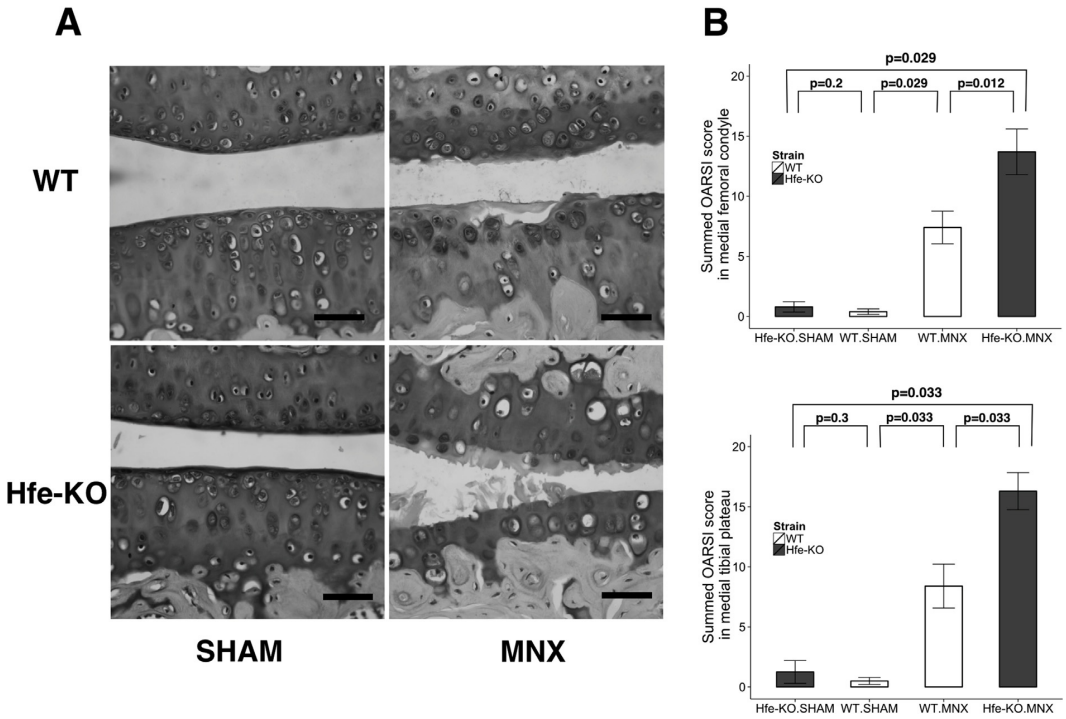

Fig. 2. Cartilage changes following meniscectomy in WT and Hfe-KO mice (A) Haematoxylin-Fast Green-Safranin-O staining of the medial femoral condyle and tibial plateau of control ($n = 10$ per group) and Hfe-KO mice ($n = 10$ per group) at 8 weeks after surgical induction of OA. Scale bar 50 μm. (B) The Osteoarthritis Research Society International (OARSI) scores for the medial femoral condyle and medial tibial plateau of control and Hfe-KO mice (SHAM and MNX operated knees) were obtained by summing the score of three sections from the lateral, middle and medial one-third of the medial knee compartment from each mouse. Data are shown as the mean (bar) and corresponding 95% CI (range).

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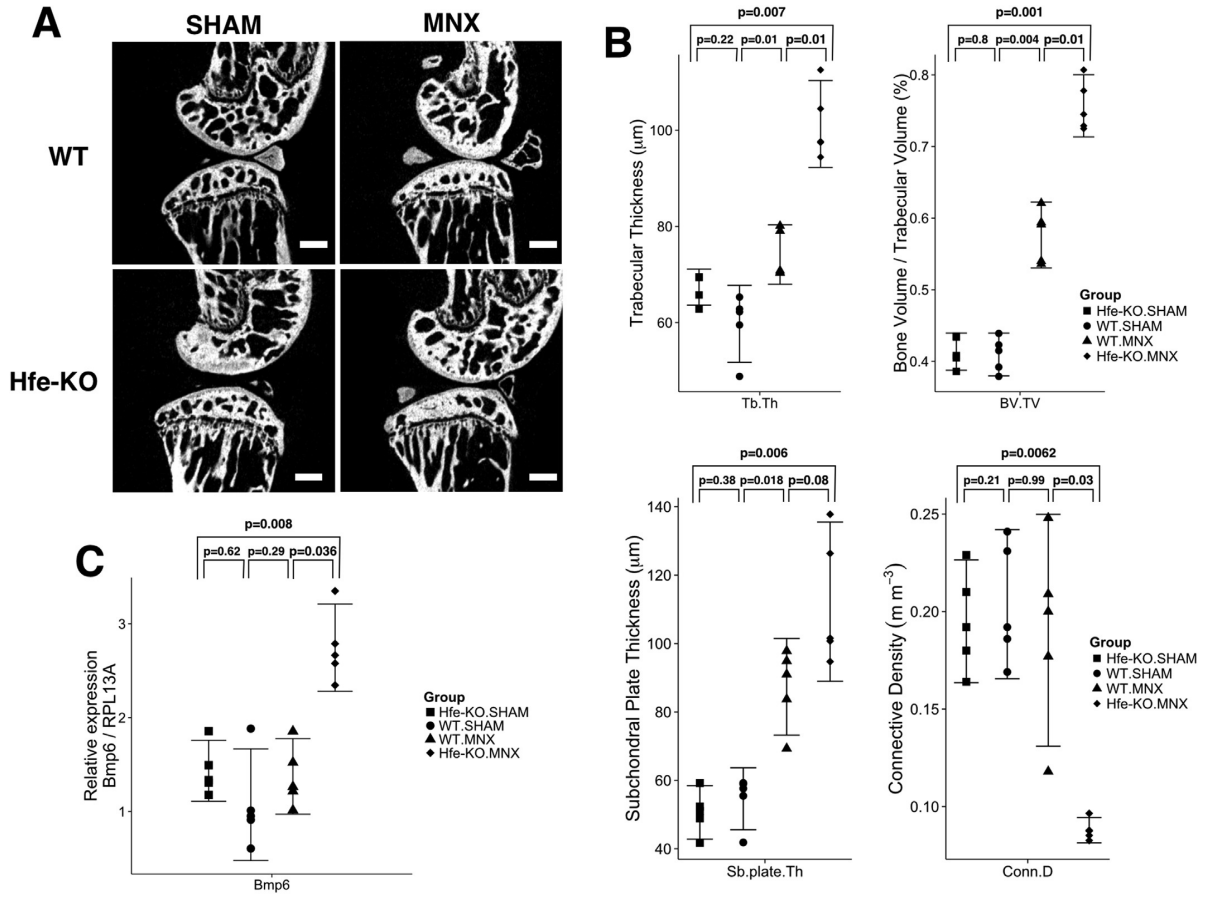

Fig. 3. Subchondral bone changes following meniscectomy in WT and Hfe-KO mice (A) Knee micro-CT sagittal reconstructions at the level of the middle one-third medial tibial plateau of control ($n = 5$ per group) and Hfe-KO mice ($n = 5$ per group) at 8 weeks after surgical induction of OA. Scale bar 500 μm (B) Quantification of microarchitecture parameters of the medial tibial plateau of mice knees ($n = 5$ per group). The results show that the MNX operated knees of the Hfe-KO animals have a greater degree of subchondral sclerosis when compared to their WT controls. Data are shown as the individual values (shapes) with the corresponding 95% CI (range) (C) Real time PCR, using mRNA isolated from the medial knee compartment of 18 weeks-old animals, to detect the expression of the *Bmp6* gene. The results show that MNX surgery leads to an increase in the expression of the gene but only in Hfe-KO mice is it significant. Data are the individual values (shapes) with the corresponding 95% CI (range).

operated knees had a similar percentage of articular chondrocytes positive for type X collagen, ADAMTS-5 and MMP-13, but the Hfe-KO, MNX operated knees displayed stronger staining and a greater percentage of MMP-3 positive cells when compared to their WT controls ($n = 3$ per group, Fig. 5).

Discussion

This is the first study describing the effect of systemic iron overload in the development of OA *in vivo*, by analysing Hfe-KO mice. At eight weeks after surgical induction of OA, the Hfe-KO mice showed an accelerated OA progression associated to a greater cartilage destruction, greater subchondral bone volume and an increased expression of a gene related to cartilage degradation when compared to their wild-type controls.

As previously described[19], the mutation had no effect on the morphology of the mice but caused a significant systemic iron overload with increased iron content observed in the serum and liver of the Hfe-KO mice. Following arthrotomy the Hfe-KO mice showed an increased deposition of iron in the form of hemosiderin in the synovial membrane. Synovial iron accumulation can also be found in human HH patients and in patients with RA[11]. It is thought that the iron arises not from the exchange with the labile iron pool but from blood that enters the joint, either by trauma or by oozing from an inflamed synovial membrane[26]. Accordingly, we hypothesise that the iron seen in the knee synovial membrane would originate from blood entering the joint following the arthrotomy. The greater hemosiderin accumulation in the Hfe-KO mice joints could be therefore explained by the higher iron content of their blood.

By itself, iron accumulation in the synovial membrane does not appear to be enough to cause cartilage damage in this time period, as shown by the data from Hfe-KO mice SHAM-operated knees, since their histological, morphological and gene expression characteristics do not show any evidence of OA and are identical to their WT counterparts. Feeding the mice with an iron-enriched diet in order to obtain an even greater iron overload or having a longer follow-up period could yield different results.

Previous reports show that the levels of proinflammatory cytokines are elevated in the first days following injury[27,28] and then gradually decrease in the following weeks, eventually returning to normal levels[29,30]. This could explain the absence of significant differences in the expression of *Il1b* and *Il6* in this study since the

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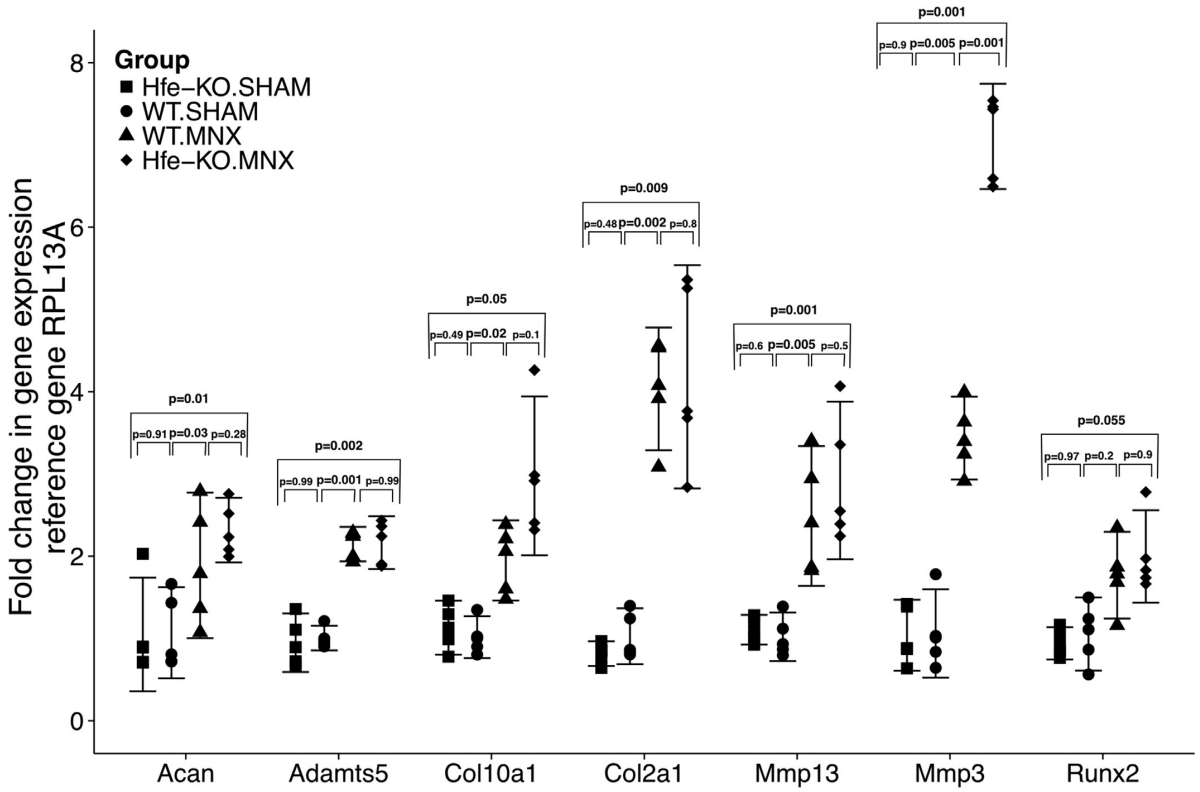

Fig. 4. Changes in gene expression following surgical induction of OA. Real time PCR, using mRNA isolated from the medial knee compartment of animals euthanized 8 weeks after surgery, to detect the expression of selected target genes ($n = 5$ per group). The results confirm that MNX surgery induces the expression of genes related with matrix degradation and chondrocyte hypertrophy and that this is more pronounced in the Hfe-KO mice. Data are shown as the individual values (shapes) with the corresponding 95% CI (range).

mice were evaluated at 8 weeks after the initial injury, when the disease process is already in a quiescent phase[31].

As previously reported in the literature, we found that surgically induced mechanical instability of the knee joint resulted in deregulation of genes related to extracellular matrix production, matrix degradation and chondrocyte hypertrophy[31–33], as well as cartilage degeneration[34,35] and subchondral bone sclerosis[36,37]. All of these were observable in both WT and Hfe-KO mice meniscectomized knees but differences were more evident in the latter (Figs. 1–4) providing further evidence for the increased susceptibility for OA in the Hfe-KO mice. On the other hand, the presence of excess iron in the synovial tissue could also account for the accelerated OA progression in the meniscectomized knees of the Hfe-KO mice since intra- and extra-cellular overload of iron in the tissue induces the generation of free radicals and reactive oxygen species (ROS)[38–40]. These can lead to hyper-activation of MMPs[41], a crucial step in many normal and pathological biological processes[42]. We observed that, following the surgical induction of OA, the Hfe-KO mice displayed an increased expression of MMP-3 when compared to their WT controls. MMP-3 is an enzyme capable of degrading several components of the extracellular matrix, making it less resilient to the mechanical stress and accelerating the progression of OA[33,43]. Therefore we hypothesise that the increased iron content in the synovial membrane contributes to an increased generation of ROS after an initial pro-inflammatory signal, such as joint trauma or surgical intervention, causing an up-regulation of MMP-3 expression, contributing to the greater cartilage damage observed in the Hfe-KO mice knees.

The precise role of subchondral bone in the initiation and progression of OA is still unclear[44,45]. A recent review[46] proposes that the non-physiologic strain on the joint causes micro damage to the subchondral bone leading to increased resorption and a decrease in thickness in the early-stages of OA, progressing to subchondral trabecular sclerosis and increased calcified cartilage thickness in the late-stage OA. These changes in subchondral bone architecture are likely to accompany cartilage damage that could, in turn, influence subchondral bone degradation, resulting in a vicious cycle. Accordingly, in our study we observed a greater increase in subchondral bone volume and calcified cartilage thickness in the joints with more pronounced cartilage damage, which was in agreement to data previously reported by Chappard *et al.*[47], Hayami *et al.*[48] and Botter *et al*[36].

The present study has some limitations. In humans, HH-related OA develops in the fourth and fifth decades of life whereas we used young mice (10-week-old) to study the development of OA therefore this model may not accurately represent the evolution of the human disease. The observed changes in cartilage and subchondral bone were restricted to a single time-point making it difficult to conclude about the entire sequence of changes leading to OA, but this could be addressed in future studies. The time point was chosen since the cartilage and subchondral bone changes are already well defined[18] and the disease process is in a quiescent phase[31]. Our selection of target genes was geared towards the main components of the extracellular matrix[49], the main proteolytic enzymes involved in matrix degradation[43,50] and the main molecules related with chondrocyte hypertrophy[51]. It is possible that

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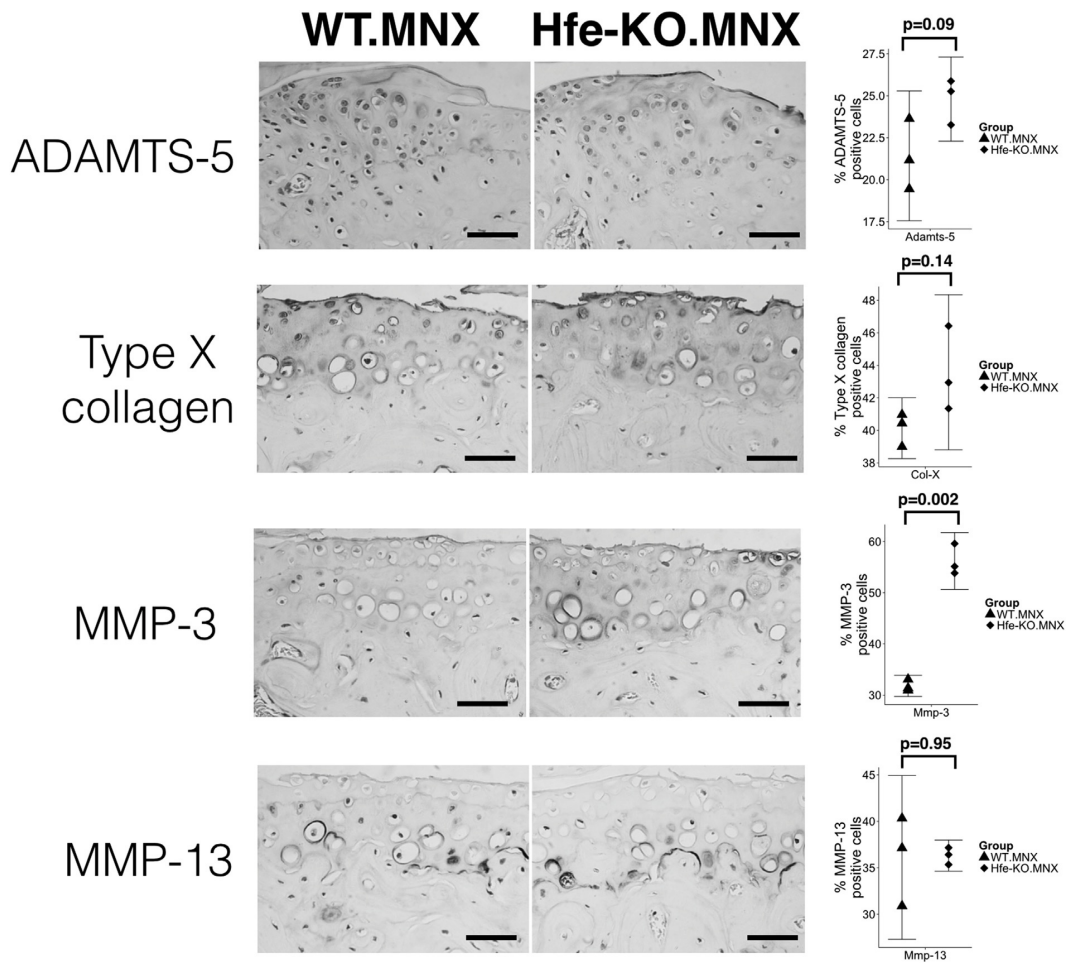

Fig. 5. Immunohistochemistry for, a disintegrin and metallopeptidase with thrombospondin motifs (ADAMTS-5), type X collagen, matrix metallopeptidase 3 (MMP-3) and matrix metallopeptidase 13 (MMP-13), in the medial tibial plateau at 8 weeks after MNX surgery in control (WT.MNX) and *Hfe*-KO (Hfe-KO.MNX) mice (3,3′-diaminobenzidine (DAB)) counterstained with Harris haematoxylin, scale bars = 50 μm). The ratio of positive cells per field was counted under a microscope at 40× magnification using three sections from three mice. The results show that MMP-3 is significantly more expressed in the Hfe-KO mice knees following MNX surgery. Data are the individual values (shapes) with the corresponding 95% CI (range).

other genes may also be relevant in the development of HH-related OA, this could also be addressed in future studies.

In conclusion, we found that HFE-KO mice are an interesting model for the study of HH-related OA since they developed accelerated knee OA progression after medial meniscectomy when compared to their WT controls, this could be secondary to the increased expression of MMP-3 observed in the iron-overloaded joints. Further studies regarding the pathway responsible for MMPs overexpression in iron-overloaded joints could provide a better insight into OA pathophysiology and lead to novel therapeutic targets.

Contributors

The seven authors are justifiably credited with authorship, according to the authorship criteria. In detail: A. Camacho – conception, design, acquisition of data, analysis and interpretation of data, drafting of the manuscript, final approval given; M. Simão – acquisition of data, analysis and interpretation of data, final approval given. H.K. Ea – acquisition of data, critical revision of manuscript, final approval given; M. Cohen-Solal – design, critical revision of manuscript, final approval given; P. Richette – critical revision of manuscript, final approval given; J. Branco – critical revision of manuscript, final approval given; M.L. Cancela – conception, design, critical revision of manuscript, final approval given. A Camacho takes responsibility for the integrity of the work as a whole.

Competing interests

None of the authors have financial or personal relationships with other people or organizations that could bias the work and conclusions stated in this manuscript.

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.joca.2015.09.007.

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Appendix B

Paper II

PLOS | ONE

RESEARCH ARTICLE

Effect of C282Y Genotype on Self-Reported Musculoskeletal Complications in Hereditary Hemochromatosis

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Competing Interests: The authors have declared that no competing interests exist.

Abstract

Objective

Arthropathy that mimics osteoarthritis (OA) and osteoporosis (OP) is considered a complication of hereditary hemochromatosis (HH). We have limited data comparing OA and OP prevalence among HH patients with different hemochromatosis type 1 (HFE) genotypes. We investigated the prevalence of OA and OP in patients with HH by C282Y homozygosity and compound heterozygosity (C282Y/H63D) genotype.

Methods

A total of 306 patients with HH completed a questionnaire. Clinical and demographic characteristics and presence of OA, OP and related complications were compared by genotype, adjusting for age, sex, body mass index (BMI), current smoking and menopausal status.

Results

In total, 266 of the 306 patients (87%) were homozygous for C282Y, and 40 (13%) were compound heterozygous. The 2 groups did not differ by median age [60 (interquartile range [IQR] 53 to 68) vs. 61 (55 to 67) years, P=0.8], sex (female: 48.8% vs. 37.5%, P=0.18) or current smoking habits (12.4% vs. 10%, P=0.3). As compared with compound heterozygous patients, C282Y homozygous patients had higher median serum ferritin concentration at diagnosis [1090 (IQR 610 to 2210) vs. 603 (362 to 950) μg/L, P<0.001], higher median transferrin saturation [80% (IQR 66 to 91%) vs. 63% (55 to 72%), P<0.001]) and lower median BMI [24.8 (22.1 to 26.9) vs. 26.2 (23.5 to 30.3) kg/m2, P<0.003]. The overall prevalence of self-reported OA was significantly higher with C282Y homozygosity than

compound heterozygosity (53.4% vs. 32.5%; adjusted odds ratio [aOR] 2.4 [95% confidence interval 1.2–5.0]), as was self-reported OP (25.6% vs. 7.5%; aOR 3.5 [1.1–12.1]).

Conclusion

Patients with C282Y homozygosity may be at increased risk of musculoskeletal complications of HH.

Introduction

Hereditary hemochromatosis (HH) is an autosomal recessive disorder characterized by increased absorption of dietary iron and rapid iron release from macrophages, which leads to abnormal accumulation of iron in several organs, particularly the liver, joints and bones [1, 2]. In patients with iron overload, 2 hemochromatosis type 1 (HFE) genotypes have been commonly described: C282Y homozygosity and C282Y/H63D compound heterozygosity. Among patients of northern European descent, the most common genotype is C282Y homozygosity, accounting for about 80% to 90% of HH cases [2].

The musculoskeletal complications of HH have been mainly described in C282Y homozygous patients. These complications consist of an arthropathy that mimics osteoarthritis (OA) and osteoporosis (OP) [3, 4]. The prevalence of OA ranges from 20% to 80% in homozygous patients with HH [4]. In addition, such patients are at increased risk of joint replacement [5]. The reported prevalence of OP in these patients ranges from 25% to 35% and OP seems to be associated with severity of iron overload [1].

Recently, there has been renewed interest in the role of HFE compound heterozygosity (C282Y/H63D) in the development of HH. Patients with the C282Y/H63D genotype show iron overload but to a lesser extent than in homozygotes, particularly in the absence of comorbid factors [6, 7]. Little is known about the risk of hemochromatosis-related musculoskeletal complications with HFE compound heterozygosity.

We aimed to compare the prevalence of HH-related OA and OP in patients with C282Y homozygosity and those with compound heterozygosity (C282Y/H63D).

Patients and Methods

Patients with HH

Patients who were members of the Association Hémochromatose France (AHF) completed a self-administered questionnaire that was previously described [8]. Patients were informed of the purpose of the study and gave their informed consent to be in the study. This study was conducted in accordance with the recommendations of the Helsinki Declaration. The Institutional Review Board (IRB00006477- Comité d'évaluation de l'éthique des projets de recherche biomédicale du GHU Nord-Hôpital Bichat, Paris) reviewed and approved the study (no. 10–074).

We collected the following data: demographic characteristics (age, sex, weight, height, current smoking habit, menopausal status); details of HH history (HFE genotype, ferritin concentration and transferrin saturation at diagnosis, symptoms before diagnosis); general clinical features (asthenia, diabetes mellitus, heart disease); joint and spine involvement (patients were asked if they had received a diagnosis of OA by a physician; if they had ever complained of low back pain or sciatica; if they had knee-, hip-, or ankle-replacement prosthesis); and bone involvement (patients were asked if they had received a diagnosis of OP by a physician; if they

had a history of fractures). To confirm the validity of the genotype reported by patients, we reviewed the medical records of a sample of 20 patients followed in our department who had reported having homozygosity (n = 10) and duplex heterozygosity (n = 10). The rate of concordance was 95% (19 of 20). One patient who declared homozygosity was actually compound heterozygous.

Statistical analysis

Continuous variables were tested for normality and homoscedasticity. Some continuous variables were non-normally distributed (skewed). Therefore, variables were described with the median and interquartile range (IQR). Differences between groups were assessed by the Mann-Whitney test. The association between categorical variables was tested by chi-square test. Crude odds ratios (ORs) were calculated by an univariate logistic regression model, with the patient genotype (C282Y/C282Y vs. C282Y/H63D) as the independent variable and the outcome of interest as the dependent variable. Adjusted ORs (aORs) were calculated by a multiple logistic regression model, with the patient genotype and clinically relevant confounders as independent variables and the outcome of interest as the dependent variable. The design of the study precluded the assessment of iron overload by liver biopsy, so we used ferritin concentration as a surrogate marker, with severity of iron overload defined by serum ferritin concentration $\geq$1000 μg/L at diagnosis. This cut-off was previously found clinically appropriate [9]. All statistical analyses involved use of Stata 13.1 (StataCorp, College Station, TX). A 2-tailed $p < 0.05$ was considered statistically significant.

Results

Demographic and clinical characteristics of patients

In total, 306 patients with HH completed the questionnaire. The sample characteristics are shown in Table 1. The median age of patients was 60 years (IRQ 53 to 68 years); 47.4% were females (80% menopausal). Overall, 266 patients (87%) were C282Y/C282Y homozygous and 40 (13%) were C282Y/H63D compound heterozygous. Median serum ferritin concentration at diagnosis was significantly higher with C282Y homozygosity than compound heterozygosity [1090 (IQR 610 to 2210) vs. 603 (362 to 950) μg/L, P<0.001], as was median transferrin saturation [80% (IQR 66 to 91%) vs. 63% (55 to 72%), P<0.001]. The proportion of patients with severe iron overload (> 1000 μg/L) was greater with C282Y homozygosity than C282Y/H63D compound heterozygosity (52.4% vs. 20%, P< 0.001).

Table 1. Characteristics of patients with hereditary hemochromatosis by genotype.

	C282Y/C282Y n = 266	C282Y/H63D n = 40	P value
Age, years	60 (53–68)	61 (55–67)	0.50
Women, %	48.8	37.5	0.18
Body mass index, kg/m^2	24.8 (22.1–26.9)	26.2 (23.5–30.3)	0.003
Menopausal women, %	77.6	80	0.80
Current smoker, %	13.2	7.5	0.30
Serum ferritin concentration at diagnosis, μg/L	1090 (610–2210)	603 (362–950)	< 0.001
Transferrin saturation, %	80 (66–91)	63 (55–72)	< 0.001

Data are median (interquartile range) or percentage. NS: not significant

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Table 2. Prevalence of self-reported symptoms at the time of the survey by genotype.

	C282Y/C282Y n = 266	C282Y/H63D n = 40	Crude OR (95% CI)	aOR*(95% CI)	P value
Joint pain, %	88.4	77.5	2.2 (0.96–5.1)	2.8 (1.2–6.9)	0.02
Asthenia, %	79.3	82.1	0.82 (0.34–1.9)	0.8 (0.33–1.9)	0.56
Diabetes, %	9.7	10	0.98 (0.32–2.9)	1.2 (0.37–3.9)	0.87
Cardiac disease, %	15.4	17.5	0.86 (0.36–2.1)	0.98 (0.39–2.5)	0.83

OR, odds ratio; aOR, adjusted OR; 95% CI, 95% confidence interval.

*Adjusted for age, sex, body mass index and current smoking habits.

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Self-reported symptoms at the time of the survey

The prevalence of self-reported joint pain at the time of the survey was significantly higher with C282Y homozygosity than C282Y/H63D compound heterozygosity (88.4% vs. 77.5%, aOR = 2.8 [95% confidence interval (95% CI) 1.2–6.9]; Table 2). The prevalence of self-reported asthenia, diabetes and cardiac disease did not differ between the genotypes.

OA, joint replacement and spine involvement

The prevalence of OA was higher with C282Y homozygosity than C282Y/H63D compound heterozygosity: 53.4% vs. 32.5% (aOR = 2.4 [95% CI 1.2–5.0]; Table 3). This difference was no longer significant on adjustment for ferritin concentration (aOR = 1.66 [0.79–3.5]). The prevalence of hip replacement was higher for homozygotes than heterozygotes–11.7% vs. 7.5% (aOR = 1.5 [0.41–5.1])—but this difference did not reach statistical significance. The prevalence of self-reported back pain and sciatica did not differ between the groups: 71.8% vs. 70% and 47.4% vs. 45%, respectively.

OP and fractures

In total, 155 patients (50.7%) reported than they had undergone dual-energy X-ray absorptiometry (DEXA). All the patients who self-reported OP also reported having undergone DEXA. The prevalence of OP was greater for C282Y homozygous than compound heterozygous patients (25.6% vs. 7.5%; aOR = 3.5 [95% CI 1.1–12.1]); Table 4). The effect of genotype on the prevalence of OP was no longer statistically significant on adjustment for ferritin concentration (aOR = 2.6 [0.74–9.3]). The prevalence of hip, wrist and vertebral fractures did not differ between the groups: 2.6% vs. 2.5%, 13.2% vs. 7.5% and 7.9% vs. 2.5%, respectively.

Table 3. Prevalence of osteoarthritis, joint replacement, back pain and sciatica by genotype.

	C282Y/C282Y n = 266	C282Y/H63D n = 40	Crude OR(95% CI)	aOR*(95% CI)	P value
Osteoarthritis, %	53.4	32.5	2.4 (1.2–4.8)	2.4 (1.2–5)	0.02
Knee replacement, %	5.6	5.0	1.1 (0.25–5.1)	1.5 (0.3–7.1)	0.64
Hip replacement, %	11.7	7.5	1.6 (0.47–5.6)	1.5 (0.41–5.1)	0.08
Ankle replacement, %	0.75	2.5	0.3 (0.03–3.3)	0.32 (0.025–4)	0.52
Back pain, %	71.8	70	1.1 (0.53–2.3)	1.2 (0.55–2.6)	0.31
Sciatica, %	47.4	45	1.1 (0.56–2.1)	1.3 (0.65–2.6)	0.59

* Adjusted for age, sex and body mass index.

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Table 4. Prevalence of osteoporosis and associated fractures by genotype

	C282Y/C282Y n = 266	C282Y/H63D n = 40	Crude OR(95% CI)	aOR*(95% CI)	P value
Osteoporosis, %	25.6	7.5	4.3 (1.3–14.2)	3.5 (1.1–12.1)	0.04
Hip fracture, %	2.6	2.5	1.1(0.13–8.8)	0.66 (0.07–6.3)	0.64
Wrist fracture, %	13.2	7.5	1.9 (0.55–6.4)	2.3 (0.65–8.2)	0.45
Vertebral fracture, %	7.9	2.5	3.3 (0.44–25.6)	3.3 (0.42–26)	0.15

* Adjusted for age, sex, body mass index, menopausal status and smoking habits

doi:10.1371/journal.pone.0122817.t004

Discussion

We investigated the prevalence of OA and OP, HH-related musculoskeletal complications, by C282Y homozygosity and compound heterozygosity (C282Y/H63D) genotype and found the prevalence of both self-reported OA and OP higher for patients with C282Y homozygosity than compound heterozygosity.

Our study population consisted of 306 patients, the C282Y/C282Y genotype being 6.7 times more frequent than the C282Y/H63D genotype, which agrees with previous studies of patients with HH-related musculoskeletal complications [10, 11]. As expected, as compared with patients with compound heterozygosity, those with the homozygous C282Y/C282Y genotype showed significantly higher ferritin concentration and transferrin saturation values, and about half of the homozygous patients showed severe iron overload. BMI was greater for compound heterozygous than homozygous patients, as was found recently [6]. Indeed, co-morbid factors such as alcohol abuse and overweight are more prevalent in C282Y/H63D than C282Y/C282Y patients [6].

In HH, abnormally increased iron absorption leads to iron loading of parenchymal cells in the liver, pancreas, heart and joints, with subsequent damage to structure and impaired function [2]. The reasons for the high frequency of the musculoskeletal system involvement in HH are unknown [2].

In this study, the 2 genotype groups did not differ in prevalence of self-reported asthenia, cardiac disease or diabetes, which is similar to findings by Waalen et al. [12], showing that patients with different genotypes of HFE self-reported similar rates of symptoms commonly associated with iron overload.

Several studies have well documented the association of HH and an arthropathy that may seriously affect quality of life [1, 8, 13]. This arthropathy can be severe, as highlighted by the high prevalence of hip, knee and ankle joint replacement in these patients [5, 8, 10].

In our study, the prevalence of self-reported OA was greater in patients with C282Y homozygosity than C282Y/H63D compound heterozygosity, after adjustment for confounding variables. The prevalence of hip replacement surgery was greater but not significantly for C282Y homozygous than compound heterozygous patients, as was found recently [14]. This increased prevalence of OA in C282Y homozygotes might be related to the more severe iron overload in these patients. Indeed, this difference was no longer significant after adjustment for ferritin concentration. Although the role of iron overload in the genesis of HH-related OA is still not fully understood, iron overload can lead to iron accumulation in the synovium and cartilage of HH patients, as seen on histology [1], and to increased ferritin concentration in synovial fluid [15]. Some of this iron can be in the form of non-transferrin–bound iron and might have deleterious effects [16] on synovial tissue, promoting inflammation with neutrophil infiltration [17] and tissue damage, as was demonstrated in the liver [18]. Iron deposits were found in synovial tissue from patients with inflammatory arthritis [1], which suggests a link between iron

content and inflammation. More recently, the serum level of vascular adhesion molecule 1 was found associated with radiographic measures of HH arthropathy, which suggests its involvement in HH joint damage [19].

Patients with HH have shown disc degeneration and calcifications in the intervertebral discs due to calcium pyrophosphate crystal deposits [20]. In a retrospective study [21], one third of patients with HH showed spine arthropathy on radiography. The literature contains no data on the prevalence of low back pain in patients with a clinical phenotype of HH. Both our genotype groups showed similar self-reported prevalence of low back pain and sciatica, which suggests that the lumbar spine involvement in HH is independent of the genotype.

We found an increased prevalence of OP, along with increased prevalence, although not significant, of vertebral fractures in homozygote patients. Again, this observation could be related to the increased iron overload observed in C282Y homozygotes. Indeed, the impact of the genotype on the prevalence of OP was no longer significant when we adjusted for ferritin concentration. Several reports have shown that HH patients, particularly males, are at increased risk of OP, which might be related to iron overload [1, 22].

The pathophysiology of OP in HH is not entirely clear. Animal and *in vitro* data support that iron excess can affect bone formation and remodeling [1]. Iron-overloaded mice show increased level of reactive oxygen species, serum tumor necrosis factor α and interleukin 6, associated with severity of iron overload [23]. HFE-/- mice show impaired bone microarchitecture and increased osteoclast number [24]. *In vitro*, iron disrupts the formation of hydroxyapatite crystals and inhibits the proliferation and differentiation of osteoblasts [1].

There are several limitations and caveats to this study. Patients were asked to remember symptoms or fractures attributed to hemochromatosis, which might suggest recall bias. As for all surveys, this study may lack robust internal validity. Because of the relatively low number of patients with compound heterozygosity, the number of rare events such as fractures or joint replacement surgery was low in this group, which might explain the non-significant findings for joint replacement and fracture. Finally, we could not differentiate symptomatic and asymptomatic fractures.

Conclusions

To conclude, this is the first study to compare the effect of genotype on HH-related musculoskeletal symptoms. C282Y homozygosity may confer increased risk of OA and OP as compared with C282Y/H63D compound heterozygosity.

Acknowledgments

We thank all the patients who participated in this survey. We are grateful to the Association Hémochromatose France, in particular Pr. H. Michel, who helped with data collection for patients with HH.

Author Contributions

Conceived and designed the experiments: PR AC SO. Performed the experiments: AC TFB PR SO. Analyzed the data: AC PR TFB MS LC SO MCS. Contributed reagents/materials/analysis tools: AC TFB PR. Wrote the paper: PR AC.

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Appendix C

Relevant authorisations

GOVERNO DE PORTUGAL | MINISTÉRIO DA AGRICULTURA, DO MAR, DO AMBIENTE E DO ORDENAMENTO DO TERRITÓRIO

dgav Direção Geral de Alimentação e Veterinária

Exm(o) (a) Senhor(a)
ANTÓNIO MANUEL MENDONÇA
ROMÃO DE BRITO CAMACHO
[redacted]

2013-06-28 017622

Nossa referência	Vossa referência	Vossa data
0421/000/000/2013		

Assunto: **PROTECÇÃO DOS ANIMAIS UTILIZADOS PARA FINS EXPERIMENTAIS E/OU OUTROS FINS CIENTÍFICOS – PEDIDO DE CREDITAÇÃO DE INVESTIGADOR(A) AO ABRIGO DA PORTARIA Nº 1005/92, DE 23 DE OUTUBRO.**

Na sequência do pedido efetuado por V. Ex.ª sobre o assunto referenciado em epígrafe, cabe-me informar que, a partir desta data, passa a ficar creditado(a) como investigador(a) ao abrigo do ponto iii), da alínea e), do nº3 da Portaria nº 1005/92, de 23 de Outubro.

Finalmente, cabe-me, também, anexar o certificado de atribuição da creditação em apreço.

Com os melhores cumprimentos.

O Diretor de Serviços

Miguel Lemos Fernandes

Anexo: Certificado

DSPA/EP

GOVERNO DE PORTUGAL | MINISTÉRIO DA AGRICULTURA, DO MAR, DO AMBIENTE E DO ORDENAMENTO DO TERRITÓRIO

dgav Direção Geral de Alimentação e Veterinária

CERTIFICADO DE ATRIBUIÇÃO DE CREDITAÇÃO

Ao abrigo do ponto i), da alínea e), do nº 3 da Portaria nº 1005/92, de 23 de Outubro, é atribuída a ANTÓNIO MANUEL MENDONÇA ROMÃO DE BRITO CAMACHO, creditação como pessoa competente (investigador(a)) para a prática de experimentação animal.

Lisboa, 25 de junho de 2013

A Diretora Geral

Maria Teresa Villa de Brito

UNIVERSIDADE NOVA DE LISBOA
Faculdade de Ciências Médicas
Comissão de Ética
(Presidente: Prof. Doutor Diogo Pais)

Decisão final sobre o projeto "Characterization of the histological and genetic profile of articular damage in a Hemochromatosis mouse model"

A Comissão de Ética da FCM-UNL (CEFCM) decidiu, por unanimidade, aprovar o projeto de investigação intitulado **"Characterization of the histological and genetic profile of articular damage in a Hemochromatosis mouse model"** (nº 11/2013/CEFCM), submetido pelo Dr. António Brito Camacho.

Lisboa, 07 de Novembro de 2013

O Presidente da Comissão de Ética,

(Prof. Doutor Diogo Pais)

TO WHOM IT MAY CONCERN

The Ethics Committee of the Faculty of Medical Sciences of the New University of Lisbon (Faculdade de Ciências Médicas da Universidade Nova de Lisboa, FCM-UNL) has unanimously approved the Research Project entitled **"Characterization of the histological and genetic profile of articular damage in a Hemochromatosis mouse model"** (nr. 11/2013/CEFCM), submitted by Dr. António Brito Camacho.

Lisbon, November 7th, 2013

The Chairman of the Ethics Committee,

(Prof. Doutor Diogo Pais)

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GOVERNO DE PORTUGAL | MINISTÉRIO DA AGRICULTURA E DO MAR

dgav Direção Geral de Alimentação e Veterinária

Ex^mo Senhor
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Universidade do Algarve
Departamento de Ciências Biomédicas e Medicina, Secção Lab 2.13
Campus de Gambelas, Ed 7, Lab 2.13
8005 – 139 FARO

2014-01-02 000003

Nossa referência	Vossa referência	Vossa data
0421/000/000 /2013		

Assunto: **PROTEÇÃO DOS ANIMAIS UTILIZADOS PARA FINS EXPERIMENTAIS E/OU OUTROS FINS CIENTÍFICOS – PEDIDO DE AUTORIZAÇÃO PARA REALIZAÇÃO DE PROJECTO DE EXPERIMENTAÇÃO ANIMAL**

Na sequência do pedido efetuado por V. Exª no sentido de poder ser autorizada a realização do projeto experimental designado **"Characterisation of the histological and genetic profile of articular damage in a Hemochromatosis mouse model"**, tendo como investigadora responsável a **Doutora Leonor Cancela**, cabe-me informar que o mesmo foi levado à consideração dos membros da Comissão Consultiva prevista na alínea b) do nº 49, da Portaria nº 1005/92, de 23 de Outubro, sendo que os mesmos não levantaram qualquer objeção à solicitação supra referida.

Mais se informa V. Exª que esta Direção Geral, depois de esclarecidas as dúvidas que a sua análise nos levantou, nada teve a opôr ao projeto apresentado, pelo que, o mesmo foi autorizado, ao abrigo do nº 8º do mesmo diploma legislativo.

Com os melhores cumprimentos,

A Diretora Geral

As) Maria Teresa Villa de Brito

DBEA/APM

Biotério
CBME / UNIVERSIDADE DO ALGARVE

UAlg
UNIVERSIDADE DO ALGARVE
DEPARTAMENTO DE CIÊNCIAS BIOMÉDICAS E MEDICINA

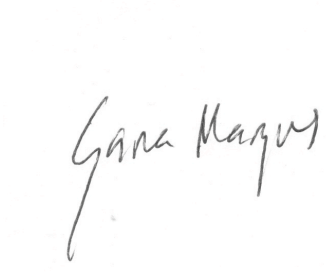
Após análise do pedido de autorização para utilização de animais com fins experimentais no Biotério da Universidade do Algarve/Centro de Biomedicina Molecular e Estrutural, e após o cumprimento das condições abaixo discriminadas, será concedida a permissão para o uso de animais em experimentação animal ao estudante António Camacho, no âmbito do seu projecto de doutoramento.

1. Numa primeira deslocação ao Biotério, será feito o acompanhamento do estudante nas instalações, e serão introduzidas as questões éticas relacionadas com o uso de animais em investigação nomeadamente as regras básicas de alojamento dos animais de modo a causar o mínimo impacto negativo aos animais, consoante a legislação em vigor, bem como as regras de utilização básicas do Biotério.

2. Durante esta visita, e outras se necessário, será assegurado que o investigador tem as competências mínimas exigidas no uso de animais experimentais.

3. Será feita a análise do projecto de investigação e da sua metodologia nomeadamente no que diz respeito à anestesia, analgesia, escolha do método de administração de substâncias/colheita de amostras, bem como a fixação de limites humanamente aceitáveis, e métodos de eutanásia.

A gestora do Biotério



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